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PRIMATES IN PESTICIDE RESEARCH

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The purpose of this presentation is to describe the Perrine Primate Research Branch of the Division of Pesticides, Bureau of Science, of the Food and Drug Administration (FDA).

Any general public health problem in the United States that may be a result of pollution of food, water, and/or air by pesticides is one that exists at a low level, with prolonged exposure. In 1965, a nonhuman primate research program was initiated by the Office of Pesticides of the USPHS to investigate any such possible problem.

The laboratory became operational a year later, at which time it was made a part of the National Communicable Disease Center. As of July, 1969 the laboratory has been a part of the FDA's recently established Division of Pesticides. It is the only primate facility in FDA.

The laboratory is located on 15 acres of land on the South Campus of the University of Miami in Perrine, Florida. Its four buildings contain a total of 50,000 square feet of floor space. Its professional staff of 33 makes use of more than $1 million worth of capital equipment and has a current operating yearly budget of more than $1 million.

The laboratory is composed of four sections: pharmacology, chemistry, training, and a field station located in Wenatchee, Washington. Only the pharmacology section is directly concerned with nonhuman primates in pesticide research, but there are many areas of overlapping interest and investigative effort among the sections.

There is a colony of 75 rhesus monkeys (*Macaca mulatta*) and a colony of 175 squirrel monkeys (*Saimiri sciureus*, Columbian and Peruvian) at the laboratory. Most of the latter are housed in outdoor, covered, wire-mesh enclosed runs. It has been observed repeatedly that the animals housed

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outside are considerably healthier than those housed indoors. Fortunately, the semitropical climate of Southeast Florida ideally lends itself to this approach. Attempts at breeding the squirrel monkeys have been successful and there were 15 live births in the spring of 1969. In addition, the laboratory maintains rat, rabbit, and dog colonies for preliminary and short-term experiments.

In the major research effort, directed at detecting effects of pesticides in nonhuman primates, investigations have been oriented toward the assessment of various biochemical and physiological parameters.

The first step, then, is to find a measurable effect. This has been somewhat handicapped by the general paucity of baseline information on nonhuman primates, and several short term experiments have been performed to provide some of these data. If an effect can be shown to be a result of the prolonged administration of pesticides at levels greater than, but approaching the level of general population exposure, the effect will be evaluated in terms of its detrimental and/or adaptive nature. In addition, the observed effect must be considered in relation to possible interactions of the pesticide with other chemical and biological stress factors, such as environmental toxicants, drugs, diseases and aging.

Subsequently, an attempt is made to relate these findings to man. In this effort the laboratory staff works with and complements the affiliated USPHS Community Studies Laboratories in their human monitoring programs. A possible effect noted in a human study population can be examined under controlled experimental conditions in the nonhuman primate; conversely, an effect noted in the monkey can be studied in man under environmental and occupational exposure conditions.

Many pesticides exert their acute toxicological manifestations through central nervous system aberrations. It is therefore presumed that the central nervous system may be a target for the effects of low-level chronic exposure as well. However, these effects will almost certainly be more subtle and difficult to observe, e.g. they might involve neuroendocrine or behavioral changes.

For neurophysiological study, experimental groups of squirrel and rhesus monkeys are given daily doses of various pesticides by feeding each animal a marshmallow into which the pesticide has been injected. The rhesus monkeys are given root beer to which doses of the pesticide have been added. These have proved very satisfactory methods of dosing. Previously, experimental groups were given daily doses with an oral dosing needle.

The pesticides administered include DDT, dieldrin, lindane, toxaphene, parathion, carbaryl, and 2,4–D. Suitable control groups have been established. The dose levels on a mg/kg/day basis are some multiple (10 to 1000 times) of the amount of pesticide consumed by the general public as ascertained by various market basket surveys. However, these levels still represent only a fraction of the reported LD_{50} of the compound.

The animals have been maintained on daily intakes of pesticide agents for from 10 to 32 months, during which time each animal has had one or more electroencephalograms. The surface EEG recordings are being obtained to derive quantitative expressions of spontaneous electrical activity in relation to pesticide exposure.

Some of these animals have been equipped with permanent stereotaxically implanted electrodes in selected hypothalamic nuclei. Both depth and surface recordings are obtained in an attempt to correlate the activity of the deeper structures with that from the surface.

Future plans include telemetry data gathering with the animals housed in open faced cages and restrained by metal harnesses. All of this electrophysiological information is being stored on magnetic tape and will be submitted to a computer analysis to determine if any characteristic changes can be correlated with exposure to specific agents.

In addition, models are being developed to study the hypothalamic-pituitary axis, with histological analysis of the former and an acrylamide gel electrophoresis hormone profile of the latter. These two techniques combined, if successful, will provide a powerful tool for evaluating possible neuroendocrine involvement with respect to pesticide exposure.

The metabolism unit of the laboratory has been investigating the possible effects of pesticides and their bio-transformation products on the intermediary metabolism of the nonhuman primate. The findings on the effects of each compound are considered in relation to their possible interaction with other compounds and/or stressful factors.

The unit has also been studying the metabolism of pesticides by the nonhuman primate. These latter 'biodynamic' properties include the absorption, transport, distribution, storage, mobilization, and excretion of the compound. They also include the identification of the compound's metabolites and metabolic pathways as well as the mechanisms involved in its degradation.

The metabolism group has used mainly squirrel monkeys to date, the majority being housed in metabolism cages so that accurate intake and output data and biological samples may be collected. Several interesting observations have been made, both on compound metabolism and on animal metabolism.

In squirrel monkeys, the red blood cell and plasma cholinesterase enzyme activity, which is depressed by the insecticide parathion, will gradually begin to recover over a period of time, even with the continued oral ingestion of the compound. Further evaluation of this observation is planned and will include study of substrate and enzyme levels, rates of metabolic degradation of parathion, and the species specificity of the finding.

The concept of the interaction of certain chemicals with liver microsomal enzyme systems is well established in rodents. It has been found that it is possible to stimulate the liver microsomal enzyme system of the squirrel monkey with agents such as DDT and DDE and that the amount of stimulation is dose related. This finding offers and additional tool for the study of the pharmacological action of organic compounds in these animals.

A study was undertaken to follow several hematological and biochemical parameters in a group of squirrel monkeys which were fed daily doses of various pesticides. In attempting to establish baseline values of several serum enzymes, a wide variation was found. It has been subsequently established that much of this variability is due to the stress of the sampling procedure. The results are quite variable at best, but if a blood sample is not collected within two minutes of the time the animal is approached, the variability is so great that it almost negates the usefulness of these parameters as indicators of altered metabolism.

What is needed, obviously, is more baseline data under various experimental conditions to better define the limits of and the reasons for the metabolic and physiologic variability in these nonhuman primates.

Future plans for the laboratory include the expansion of the primate facility so that there are more single-caged monkeys, more outdoor shelters, a facility for programmed reproduction of primates, and a large free-ranging arena in which a baboon colony may be established and telemetry data gathering facilitated. And, finally, the future may bring an expansion of our central theme of study to include research in other areas of interest to the FDA.

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Poisoning by DDT: Relation between Clinical Signs and Concentration in Rat Brain

Abstract. *The severity of signs of poisoning in rats after a single dose of DDT is directly proportional to the concentration of the compound in their brains. The concentrations associated with death after one large dose are about the same as those after many smaller doses.*

The action of DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane] in animals is manifested almost entirely through the nervous system. The most prominent signs of poisoning from this compound are muscle tremor, incoordination, and convulsions. Studies of the effect of DDT on the nervous system have been reviewed elsewhere (*1*). Briefly, early studies in which measurements were made of DDT in brain and other tissues failed to relate the concentrations found to clinical signs of poisoning. In more recent work, Dale *et al.* (*2*) showed that the concentration of DDT in the brain of rats fed the *p,p′*-isomer of DDT, at a dietary level of 200 parts per million for 90 days, increased during a subsequent 10-day period of partial starvation. The increased concentration of DDT in the brain during starvation was correlated with the clinical signs of poisoning.

This report gives the concentration of DDT and DDE [1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene] in rats before, during, and after recovery from signs of illness following a single oral dose of DDT. The results show that clinical signs of poisoning are directly correlated with the concentration of DDT in the nervous system, as measured by the concentration in the brain. Special studies have shown that all parts of the nervous system are affected by DDT. However, a review (*1*) of published papers, and new research to be published elsewhere, indicates that the brain is of major importance in this regard.

Forty male rats, 100 days old, of the Sherman strain, each weighing about 240 to 300 g, were kept in separate cages and given a single dose of 3-percent *p,p′*-DDT in peanut oil solution, by stomach tube, at a rate of 150 mg/kg of body weight. The rats had been reared on Purina laboratory chow with no DDT added, but were fasted overnight before being dosed with DDT.

Surviving rats were observed continuously for symptoms of poisoning during the first 12 hours after being dosed. They were killed according to the following schedule: group 1 (asymptomatic), 2 hours after dosing; group 2, at the onset of slight tremor; group 3, after 2 to 4 hours in severe tremor; group 4, at the onset of convulsions; and group 6, 26 hours after dosing, following observed severe tremor or convulsions, or both, with subsequent complete clinical recovery. Group 5 consisted of rats that died during or just after convulsions; samples were taken as in the other groups. The rats were assigned to their respective groups at the time of killing or death, and the brain, liver, kidneys, blood, and a fat specimen were taken for chemical analysis.

Since the rats in groups 1 to 4 were killed before or while they were showing signs of poisoning, there was no way to separate those that might have survived from those that might have died.

Blood was collected from living rats by cardiac puncture, while the rats were under ether anesthesia. After as much blood as possible was withdrawn, the rats were bled to death by severing the aorta. Blood for analysis was taken from the heart of dead rats within a few minutes after they died. Heparin was used in the syringe to prevent clotting.

The plasma was extracted by the method of Bloor (*3*). The DDT and DDE in the plasma extract were measured by the Schechter-Haller method, as modified by Schechter *et al.* (*4*). The brain, liver, kidneys, and fat tissues were analyzed for DDT and DDE by the Schechter-Haller method as modified by Mattson *et al.* (*5*).

No clinical signs of poisoning were observed in the rats during the first 2 hours after being given the single oral dose of DDT. Some of the rats began to show slight tremor after 3 hours, severe tremor after 4.5 hours, and convulsions after 5.4 hours of being dosed. All of the rats that developed convulsions were in severe tremor when the convulsions began.

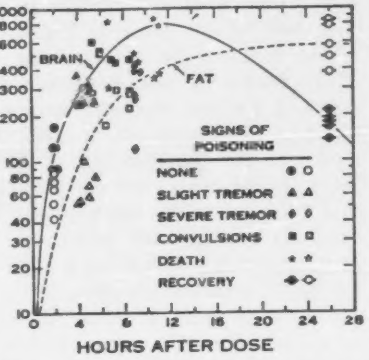

Fig. 1. Concentration of DDT in the lipids of brain (solid symbols) and fat (open symbols) of rats at different times after the administration of a single dose of the compound at the rate of 150 mg/kg. Different symbols indicate the clinical state of the animals when the samples were taken.

The concentrations of DDT and DDE found in the extractable lipids of the different tissues are shown in Table 1. It can be seen that there was a significant increase in the concentration of DDT in the brain of the poisoned rats which corresponded with the increase in the severity of the symptoms, but there was no significant difference between the concentrations of DDT in the brains of rats killed in severe tremor and those killed in convulsions. The concentration of DDT increased significantly in the plasma and kidneys until the onset of slight tremor, and in the liver until the time of severe tremor. There was no further increase in the concentration of DDT in the plasma and liver of the rats at the time of severe tremor, convulsions, or death. The sharp increase in the concentration of DDT in the kidneys of the rats that died as compared with the concentration in those killed after convulsions ($p < .001$) may have been due to renal failure.

Animals in group 6 were killed 26 hours after being dosed with DDT, when they were no longer showing signs of poisoning. The concentration of DDT in their brains had returned to levels below that of any rat showing toxic symptoms, and the concentrations in the plasma, liver, and kidneys had decreased also. However, the DDT in fat had continued to increase as it was mobilized from other tissues. The temporal relations of the changes in the brain and fat are shown in Fig. 1.

The major signs of poisoning by DDT concern the central nervous system, and the data presented here suggest that these signs are a direct response to the concentration of DDT found in the brain. In the rats that died (group 5), the concentration in the brain (524 to 848 ppm) was in good agreement with the range (205 to 985 ppm) reported elsewhere (*2*) for rats that died promptly when subjected to partial starvation after receiving small doses of DDT over long periods. This suggests that, regardless of how the DDT is administered, the probability that death will occur increases if the concentration of DDT in the brain exceeds 500 ppm in otherwise healthy rats, or exceeds 200 ppm in debilitated rats.

These data confirm the known fact that the concentration of DDT in the fat is not related to acute toxicity.

The appearance of measurable amounts of DDE in the plasma before other tissues parallels the results of Rothe *et al.* (*6*) who found that DDE appeared in chyle collected from the ductus lymphaticus during the absorption of DDT from the intestine. Values for DDE in brain, liver, kidneys, and fat are not shown in the table, because the concentrations were all below the experimental limits of the method.

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Table 1. The concentration of DDT and DDE in tissue lipids of male rats at various time intervals and in various clinical states after administration of a single oral dose of DDT at the rate of 150 mg/kg. The results are presented as the range, mean, and standard error.

No. of rats	Time between administration of DDT and death (hr)	DDT (ppm)					DDE (ppm)
		Brain*	Plasma	Liver	Kidney	Fat	Plasma
Group 1: no clinical signs of poisoning							
6	2	90 – 168†	178 – 417	737 – 1074	286 – 1002	29 – 84	<0.5‡
	2	119 ± 14	268 ± 40	947 ± 58	597 ± 126	58 ± 8	
Group 2: slight tremor							
6	4.00 – 5.58	242 – 371	178 – 1190	1706 – 4626	1029 – 1894	54 – 99	<0.5‡
	4.75 ± 0.23	287 ± 21	833 ± 147	2839 ± 470	1495 ± 130	68 ± 7	
Group 3: severe tremor							
3	9.00 – 9.50	386 – 433	215 – 1086	4602 – 6657	1030 – 1211	118 – 266	99 – 193
	9.17 ± 0.17	404 ± 15	645 ± 251	5818 ± 622	1100 ± 56	213 ± 47	129 ± 32
Group 4: convulsions							
6	5.50 – 8.50	289 – 606	643 – 1348	2420 – 7609	1341 – 1597†	173 – 512	<0.5 – 145
	7.05 ± 0.55	468 ± 43	1007 ± 114	4149 ± 734	1443 ± 45	293 ± 47	98 ± 21‡
Group 5: convulsions and death							
4	6.80 – 11.25	524 – 848	417 – 893	4270 – 6602	2885 – 3994†	309 – 424	298 – 476
	9.06 ± 1.12	737 ± 73	685 ± 107	5698 ± 500	3315 ± 343	361 ± 24	372 ± 45
Group 6: recovery after severe tremor or convulsions, or both							
5	26	138 – 213	119 – 178	755 – 1162	591 – 871	377 – 810	298 – 476
	26	176 ± 12	131 ± 12	958 ± 82	720 ± 51	598 ± 82	405 ± 34

* *p*-Values: group 1 versus 2, $< .001$; 2 versus 3, $< .01$; 3 versus 4, $> .2$; 4 versus 5, $< .01$; 2 versus 6, $< .005$. † One sample lost. ‡ In calculating the mean and standard error, values below the detectable limits (0.5 ppm) were counted as zero.

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Chlorinated Insecticides and Related Materials in Human Urine

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There is limited knowledge on the excretion of neutral chlorinated hydrocarbon insecticides and their metabolites in human urine. Such materials have not been reported in the urine of people without special exposure to insecticides. However, urinary excretion of dieldrin and its metabolites has been measured in men with occupational exposure to this insecticide (Cueto and Hayes, 1962).

The levels of excretion of chlorinated insecticides and related materials in the urine of the general population as well as occupationally exposed individuals would be of pharmacologic interest, and these levels may possibly serve as exposure indices. Furthermore, urine samples are easier and less painful to secure than blood samples, particularly where a number of tests must be made on the same individual over a period of time. This paper describes a method for the detection of neutral chlorinated hydrocarbon insecticides in human urine.

METHODS

The chemical investigation that led to the following method is described in the section on Results under the heading "Factors Influencing the Analyses." Urine (2 ml) was placed in a 25-ml graduated cylinder and adjusted to a pH of 7.0 with either 2% aqueous sodium hydroxide or 0.5 *N* phosphoric acid. Acetonitrile (4 ml) was added to the tube. To this homogeneous solution was added 10 ml of water that had been extracted previously with *n*-hexane. The entire solution was then extracted three times with 2-ml portions of *n*-hexane. The extracts were combined in a calibrated centrifuge tube and made up to a volume of 6.0 ml. The *n*-hexane extract was then dried by adding approximately 0.2 g of anhydrous sodium sulfate and shaking the tube on a Minimixer.[2] A 5-ml aliquot, equivalent to 1.67 ml of the original urine specimen, was pipetted into a 15-ml centrifuge tube, which was then placed in a constant temperature bath maintained at 40°. The *n*-hexane was removed with the aid of a gentle stream of clean, dry air. The residue was taken up in 100 μl of hexane saturated with methanol (3 : 1, v/v). A 10-μl aliquot, equivalent to 0.167 ml of the original urine specimen was analyzed by means of gas chromatography.

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[2] Fisher Scientific Company, Atlanta, Georgia.

The instrument used was a gas chromatograph[3] equipped with a ^{63}Ni electron-capture detector and a 1-mv strip-chart recorder.[4] Quantitative analysis of component chlorinated hydrocarbon insecticides of each sample was effected by comparing the peak heights with those obtained from a chromatogram of a mixed insecticide standard of known concentration. Confirmation of the identity of the chlorinated insecticides was made by comparing the behavior of selected samples on three different columns. Column I consisted of an aluminum tube, 6 feet × ¼ inch o.d. packed with 3% QF-1[5] on 70/80 mesh acid-washed Chromosorb "G."[6] Column II consisted of an aluminum tube, 4 feet × ¼ inch o.d. packed with 2% Dow 200 oil[7] on 70/80 mesh dimethyldichlorosilane-treated Chromosorb "G." Column III consisted of an aluminum tube, 6 feet × ¼ inch o.d. packed with 3% diethylene glycol succinate on 60/80 mesh acid-washed Chromosorb "G." The three columns were operated under similar conditions: inlet and outlet blocks, 245°; column oven, 180°; electron-capture detector, 290°; carrier gas, 5% methane and 95% argon at 75 ml/min; and power source, pulse mode of operation at 45 volts. Relative retention times for the chlorinated hydrocarbon insecticides under consideration are listed in Table 1. All results reported in this paper were obtained from column I. Columns II and III were used merely for qualitative confirmation.

TABLE 1

RELATIVE RETENTION TIMES OF ELEVEN CHLORINATED HYDROCARBON INSECTICIDES ON GAS CHROMATOGRAPHIC COLUMNS

Insecticide	Relative retention times[a]		
	Column I[b]	Column II[b]	Column III[b]
α-BHC	0.29	0.18	0.40
γ-BHC	0.37	0.23	0.59
β-BHC	0.45	0.20	1.81
δ-BHC	0.50	0.23	1.44
o,p'-DDE	0.75	0.81	0.78
Heptachlor epoxide	0.91	0.65	0.84
p,p'-DDE	1.00	1.00	1.00
o,p'-DDT	1.29	1.33	1.36
Dieldrin	1.45	1.00	1.23
p,p'-DDD	1.80	1.33	2.50
p,p'-DDT	1.95	1.88	3.08

[a] Relative to the retention time of *p,p'*-DDE as 1.00. The actual retention times of *p,p'*-DDE on the respective columns were: column I, approximately 22.4 minutes; column II, approximately 16.0 minutes; and column III, approximately 9.2 minutes.

[b] The columns are described in the text under Methods.

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[3] Micro-Tek GC 2503 R, Micro Tek Instrument Corporation, Baton Rouge, Louisiana.

[4] Westronics, Inc., Fort Worth, Texas.

[5] This is a fluorosilicone obtained from Analabs, Inc., Hamden, Connecticut.

[6] Johns Manville, New York, New York.

[7] This is a silicone oil obtained from Analabs, Inc., Hamden, Connecticut.

RESULTS

Excretion of Chlorinated Hydrocarbon Insecticides in the General Population

A total of ten isomers or metabolites of chlorinated hydrocarbon insecticides were detected in the urine specimens examined in the present work, including three isomers of BHC, *p,p′*- and *o,p′*-DDT, *p,p′*- and *o,p′*-DDE, *p,p′*-DDD, heptachlor epoxide, and dieldrin. Data based on column I reported in Table 2 indicate the concentrations of materials excreted in the urine from 5 men and 5 women of the general population with no occupational exposure to chlorinated insecticides. There was no significant difference between males and females in the

TABLE 2

CONCENTRATION OF CHLORINATED HYDROCARBON INSECTICIDES IN THE URINE OF FIVE MEN AND FIVE WOMEN OF THE GENERAL POPULATION AS DETERMINED BY ELECTRON-CAPTURE GAS CHROMATOGRAPHY

Compound	Males (ppm)[a]	Females (ppm)[a]	Significance of difference (*P*)
p,p′-DDT	0.0070 ± 0.0023 0.0018–0.0138	0.0063 ± 0.0004 0.0054–0.0073	>0.10
o,p′-DDT	0.0010 ± 0.0001 0.0007–0.0011	0.0018 ± 0.0001 0.0013–0.0021	>0.50
p,p′-DDE	0.0156 ± 0.0015 0.0114–0.0209	0.0219 ± 0.0025 0.0144–0.0285	>0.10
o,p′-DDE	<0.0001–0.0008 4 positive	<0.0001–0.0010 2 positive	—
p,p′-DDD	0.0031 ± 0.0008 0.0008–0.0067	0.0030 ± 0.0003 0.0024–0.0038	>0.50
α-BHC	0.0003 ± 0.0001 0.0001–0.0005	0.0004 ± 0.0001 0.0002–0.0005	>0.40
γ-BHC	<0.0001–0.0002 2 positive	<0.0001–0.0004 4 positive	—
δ-BHC	<0.0001–0.0009 1 positive	<0.0001–0.0004 1 positive	—
Heptachlor epoxide	<0.0001–0.0005 1 positive	<0.0001–0.0009 4 positive	—
Dieldrin	0.0008 ± 0.0002 0.0005–0.0014	0.0013 ± 0.0001 0.0011–0.0019	>0.50

[a] The mean ± SE and range are given. In those instances in which some samples were negative, no mean can be given; however, the number of positive samples is indicated.

concentration of the ten compounds for which statistical evaluation could be made ($P > 0.10$). The identity of each material detected was confirmed by the use of the other two chromatographic columns described in the section on Methods. All the compounds listed in Table 1 would have been detected if they had been present in concentrations of 0.0001 ppm or more. Reagent blanks run concurrently with the samples at frequent intervals did not result in elevated baselines or any unidentified peaks within the pertinent range of the chromatogram.

Excretion of Chlorinated Hydrocarbon Insecticides in Occupationally Exposed Individuals

A critical test of the validity of associating a chromatographic peak with a particular substance is that the heights of the peak for a series of specimens show an appropriate correspondence with the exposure levels of the men (or animals) from whom the specimens were taken. It was possible to make this test in the present study in connection with dieldrin and DDT. Specimens of urine were available from men in a factory where dieldrin and aldrin are made and formulated. Similar samples were available from other men in a completely separate factory where DDT is made and formulated.

Statistical evaluation of the data obtained for dieldrin (Table 3) indicated a significant difference in concentration of excreted dieldrin among each of the three groups examined and between the general population and men with low occupational exposure (high versus medium, $P < 0.025$; medium versus low, $P < 0.005$; low occupational versus general population, $P < 0.025$). Even those with the highest exposure to dieldrin showed no significant change in their excretion of other insecticides or their metabolites.

Unlike the situation for dieldrin, extensive environmental exposure to DDT resulted in an insignificant increase in urinary excretion of DDT or its neutral metabolites (Table 3). (There was, as will be described elsewhere, a significant

TABLE 3

SUMMARY OF AVERAGE CONCENTRATIONS OF DIELDRIN AND DDT-DERIVED MATERIALS IN THE URINE OF MEN OF THE GENERAL POPULATION AND OF MEN WITH DIFFERENT DEGREES OF OCCUPATIONAL EXPOSURE TO DIELDRIN OR DDT

			Concentration of chlorinated hydrocarbon (ppm)			
Exposure group	Degree of exposure	Number of samples	Dieldrin	p,p'-DDT	p,p'-DDE	Total DDT
General population	Trace	5	0.0008	0.0070	0.0156	0.0244
Occupational exposure						
Dieldrin	Low	5	0.0053	0.0043	0.0133	0.0190
	Medium	4	0.0138	0.0077	0.0161	0.0256
	High	5	0.0514	0.0073	0.0172	0.0265
DDT	Extensive	35	0.0023	0.0113	0.0212	0.0349

increase in the excretion of the acid metabolite DDA.) Men who formulated DDT did not show significant change in the excretion of other insecticides or their metabolites except dieldrin, for which a significant ($P < 0.05$) but unexplained difference was found.

Details of the excretion and storage of insecticides in men with occupational exposure will be reported separately.

Efficiency of the Technique

The efficiency of the extraction technique was tested by adding standard materials in amounts corresponding to approximately the same concentration of *p,p'*-DDE present in the control urine specimen. The standard hexane solution of mixed chlorinated hydrocarbon insecticides was evaporated to dryness with a gentle stream of clean, dry air. To each tube was added 2 ml of the control urine sample, and the contents of the tube then were shaken and allowed to stand for approximately 1 hour. The urine was extracted by the procedure described in the section on Methods. The data shown in Table 4 indicate the recoveries obtained at the various concentrations of insecticides. These recoveries ranged from a low of 2.3% for α-BHC at a concentration of 0.20 ppm to a high of 106% for *p,p'*-DDE at a concentration of 0.04 ppm. The proportion of α-, γ-, and β-BHC recovered tends to decrease with increasing amounts added. In the majority of trials with other insecticides and related materials, however, recovery was 80% or better.

TABLE 4

RECOVERY OF CHLORINATED HYDROCARBON INSECTICIDES ADDED TO SUBSAMPLES OF A CONTROL URINE SPECIMEN

Insecticide	Recovery of added concentrations (%)			
	0.02 ppm	0.04 ppm	0.10 ppm	0.20 ppm
_p,p'_-DDT	81.0	102.0	72.0	66.0
_o,p'_-DDT	90.0	101.5	81.6	75.5
_p,p'_-DDE	84.5	106.0	86.1	82.0
_o,p'_-DDE	80.5	101.2	69.9	72.0
_p,p'_-DDD	93.0	63.0	93.6	81.0
α-BHC	25.5	18.0	7.5	2.3
β-BHC	99.5	96.5	49.9	36.6
γ-BHC	34.5	39.2	17.4	11.9
δ-BHC	87.0	104.0	81.6	73.5
Heptachlor epoxide	74.5	99.3	70.8	70.0
Dieldrin	73.5	104.0	78.1	66.5

Factors Influencing the Analyses

The effectiveness of various solvents, pH levels, conditions of hydrolysis, and a volatilization procedure was explored.

The extraction procedure described in the section on Methods represents the simplest and most efficient one found for extracting chlorinated insecticides from human urine. Several other procedures were attempted in the search for a maximal recovery of insecticide. Simple hexane extraction as well as acetonitrile extraction of a urine specimen saturated with crystalline sodium chloride was attempted. The latter procedure led to extensive interference when the extract was chromatographed. The interfering materials generally could be eliminated by adding water to the residue after the acetonitrile had been evaporated and extracting the solution with hexane. The technique described in the section on Methods represents an adaptation of this procedure.

Anticipating the probability that the chlorinated insecticides and their metabolites would be excreted as conjugates (Cueto and Hayes, 1962; Jensen *et al.*, 1957), possibly of amino acids (Pinto *et al.*, 1965), we investigated the effect of variation of pH of the urine and the effect of hydrolysis on their recovery. Optimal recovery was obtained when the urine was adjusted to a pH of 7.0.

Hydrolysis of the urine by refluxing the specimen with added dilute sodium hydroxide or dilute sulfuric acid for 1 hour did not increase the recovery of insecticide. The effect of hydrolysis at autoclave pressures and temperatures was also examined in the hope of increasing recovery. Hydrolysis of the urine (a) with dilute sodium hydroxide (pH, 9.0), or (b) with dilute sulfuric acid (pH, 3.0), or (c), for urine adjusted to pH 7.0, by autoclaving at 22 psi and a temperature of 112° for 1 hour did not result in increased recovery of insecticide.

A recently developed volatilization technique (Dale *et al.*, 1967) has been found to increase the recovery of insecticides from whole blood. This method involves a preextraction volatilization step in which the blood specimen is heated in a special test tube at 125° for 5 minutes in an aluminum block. This procedure was attempted in order to determine the effect of volatilization on recovery of chlorinated insecticides from human urine, but no improvement was observed. Additional hydrolysis experiments involved heating a small quantity of urine (5 ml) in a centrifuge tube at 95° for 15 minutes. In none of these experiments was the recovery of insecticides from a control urine specimen improved. Table 5 summarizes the recovery of *p,p′*-DDT, *p,p′*-DDE, and dieldrin from the control urine sample utilizing the various techniques just described. Entry 10, Table 5 indicates the best overall recovery of insecticide obtained from the control urine specimen. Recovery of dieldrin by means of a hexane extraction was 0.0067 ppm (entry 9) compared with 0.0043 ppm by the procedure described in the section on Methods (entry 10). The recoveries of *p,p′*-DDE and *p,p′*-DDT, however, were considerably lower. Entry 5 indicates an improved recovery of *p,p′*-DDE when autoclave hydrolysis is used at a urine pH of 3.0. As can be seen, the recovery of the two remaining insecticides is somewhat lower than that obtained with the established procedure (entry 10).

A series of experiments designed to improve recovery of insecticide from the residue after the extract solvent had been evaporated was also performed. The residual chlorinated insecticides were generally taken up in 100 μl of solvent; of this 10 μl was analyzed by means of gas chromatography. Table 6 lists the recoveries obtained from the solvents used in the investigation. Re-

TABLE 5

COMPARISON OF RECOVERIES OF *p,p'*-DDT, *p,p'*-DDE, AND DIELDRIN FROM A CONTROL URINE SPECIMEN SUBJECTED TO VARIOUS TREATMENTS PRIOR TO EXTRACTION[a]

	Pretreatment		Recovery (ppm)		
Entry	pH	Procedure	*p,p'*-DDT	*p,p'*-DDE	Dieldrin
1	7.0	Reflux[b]	0.0011	0.0061	0.0004
2	3.0	Reflux[b]	0.0022	0.0096	0.0005
3	9.0	Reflux[b]	0.0001	0.0011	0.0002
4	7.0	Autoclave[c]	0.0028	0.0097	0.0006
5	3.0	Autoclave[c]	0.0017	0.0235	0.0023
6	9.0	Autoclave[c]	0.0046	0.0212	0.0022
7	7.0	Volatilization[d]	0.0059	0.0187	0.0009
8	7.0	Volatilization[e]	0.0064	0.0057	0.0025
9	—[f]	None	0.0014	0.0093	0.0067
10	7.0	None	0.0075	0.0225	0.0043
11	3.0	None	0.0045	0.0175	0.0041
12	9.0	None	0.0048	0.0160	0.0026

[a] The extraction procedure described in the section on Methods was used except where otherwise indicated.

[b] Samples of urine were refluxed for 1 hour after appropriate pH adjustments had been made (see text).

[c] Samples of urine were autoclaved at 22 psi and 112° after appropriate pH adjustments had been made (see text).

[d] Urine (5 ml) was heated at 95° for 15 minutes in an aluminum block.

[e] Urine (0.5 ml) was heated at 125° for 15 minutes in an aluminum block.

[f] Direct hexane extraction with no adjustment of pH or any other intermediate procedure.

TABLE 6

COMPARISON OF RECOVERIES OF *p,p'*-DDT, *p,p'*-DDE, AND DIELDRIN FROM RESIDUE OF EVAPORATED SAMPLE EXTRACT USING SEVERAL SOLVENT SYSTEMS

		Recovery (ppm)		
Entry	Solvent system	*p,p'*-DDT	*p,p'*-DDE	Dieldrin
1	Hexane–0.5% acetone	0.0021	0.0069	0.0005
2	Hexane–2.0% acetone	0.0008	0.0040	0.0006
3	Hexane–0.5% isopropanol	0.0023	0.0036	0.0005
4	Hexane–2.0% isopropanol	0.0023	0.0132	0.0003
5	Hexane saturated with acetonitrile[a]	0.0021	0.0084	0.0010
6	Hexane saturated with methanol (3 : 1, v/v)[b]	0.0067	0.0216	0.0023
7	Hexane	0.0015	0.0038	0.0004

[a] The extraction procedure described in the section on Methods was used.

[b] The saturated hexane (upper) layer was used.

coveries are given for *p,p'*-DDT, *p,p'*-DDE, and dieldrin using a control urine sample. The data presented in the table indicate that optimal recoveries were obtained by using hexane saturated with methanol (3 : 1, v/v), and this solvent system was subsequently used in all operations. Several other solvent systems that were tried are not listed in the table. They generally led to elevated baselines and interfering peaks.

DISCUSSION

Investigations of the urinary excretion of chlorinated insecticides and their metabolites in humans have been limited almost exclusively to occupational exposure cases involving DDT (Durham *et al.*, 1965a) or dieldrin (Cueto and Hayes, 1962) or to controlled-dosage experiments with DDT or its analogs (Neal *et al.*, 1946; Smith, 1946; Winteringham *et al.*, 1950). The levels of excreted DDA have, in fact, been used as an exposure index to DDT (Durham *et al.*, 1965a). The presence of unmetabolized DDT in the urine has never been proved before. Incidentally, the finding of traces of it does not contradict the statement (Neal and von Oettingen, 1946; Neal *et al.*, 1946) that concentrations detectable colorimetrically do not occur even in men with occupational exposure. The presence of 10 isomers or metabolites representing DDD, BHC, aldrin, dieldrin, and heptachlor has now been demonstrated in human urine. These results are consistent with the finding that levels of chlorinated insecticides are found stored in the fat and circulating in the blood of humans. The qualitative accuracy of the findings is demonstrated by similarity of results with three chromatographic columns and, in the case of dieldrin, by greater excretion in men with occupational exposure to dieldrin.

The high levels of *p,p'*-DDE found to be excreted in the urine of people in the general population may be significant and may explain the equilibrium of storage that occurs in humans. The measured excretion of DDE accounts for about 0.02 mg/man/day, or about half of the measured current daily intake of DDT (Durham *et al.*, 1965b).

SUMMARY

A new gas chromatographic method for the analysis of chlorinated hydrocarbon insecticides and their neutral metabolites in human urine is presented. Good reproducibility and recovery are obtained. In the majority of trials, recovery was 80% or better. The method was applied to urine specimens from men and women of the general population and has resulted in the detection of ten isomers or metabolites of chlorinated hydrocarbon insecticides. The excretion of these materials in the urine of individuals with no occupational exposure to chlorinated insecticides has not been detected previously. It is undoubtedly the extremely high sensitivity of the electron-capture detector to chlorinated hydrocarbon insecticides and the good reproducibility of measurements made in this way that make it possible to establish the excretion levels demonstrated in this work.

That the materials reported were accurately identified was demonstrated by similarity of results with three chromatographic columns and, in the case of dieldrin, by greater excretion in men with occupational exposure to the compound. Occupational exposure to DDT does lead to an increase in excretion of DDA, but not to significant increase of excretion of neutral materials related to DDT. Whether measurement of urinary excretion can be used effectively to establish the degree of exposure to chlorinated hydrocarbon insecticides for any given individual remains to be seen. Clearly, additional investigation is warranted.

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Chlorinated Insecticides in the Body Fat of People in the United States

plus 8 more

Abstract. *Benzene hexachloride and dieldrin are present in the body fat of people in the general population of the United States. The mean concentration of dieldrin is 0.15 ± 0.02 parts per million, which is in good agreement with the concentration reported for southern England. The mean concentration of benzene hexachloride is 0.20 ± 0.04 ppm, which is considerably lower than the mean concentration reported for France. Paired analyses of DDT by gas chromatographic and colorimetric methods show that the results of the latter may give incorrectly high results when applied to human fat.*

Both DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane] and its metabolite DDE [1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene] are present in the body fat of people in the general population of the United States (*1, 2*) and other countries (*3–5*). It was shown recently, by the use of gas chromatography and highly sensitive detectors, that dieldrin (*4*) (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-*endo-exo*-5,8-dimethanonaphthalene) and benzene hexachloride (*5*) (1,2,3,4,5,6-hexachlorocyclohexane), respectively, are present in the body fat of people in southern England and France. No information has been available as to the occurrence of chlorinated pesticides, other than DDT and DDE, in the body fat of the general population of this country, but, because the average concentrations reported for DDT and DDE in the United States (*2*) were higher than those reported for other countries (*3–5*), we suspected that other chlorinated pesticides might also be present. Therefore, in connection with a periodic survey carried out in 1961–62 to determine whether there has been any change in the storage of DDT in people of this country, those samples of fat that were large enough were analyzed not only by the Schechter-Haller method (*6*) but also by microcoulometric gas chromatography (*7*) to detect other insecticides.

The results obtained by the Schechter-Haller method (*6*) for DDT and DDE in 131 samples from the general population and 51 other samples from people with occupational or other special exposure are to be reported separately (*8*). The two autopsy and 28 surgical samples reported here (Table 1) were collected in Wenatchee, Washington; Phoenix, Arizona; and Louisville, Kentucky. All but four of the subjects from whom the samples were obtained had been residents of the respective states at least 2 years; only two (samples 29 and 30) had experienced occupational exposure to pesticides. In no instance was illness related to pesticides.

For this study, samples of body fat (18 to 42 g) were taken for comparative study by Schechter-Haller colorimetric analysis, and by gas and paper chromatography. These samples were chopped and extracted in a Waring blender with *n*-hexane. After extraction was complete, sodium sulfate was added and mixing was continued for 5 minutes to remove moisture. The extracts were then filtered and given a preliminary cleaning by partitioning with acetonitrile (*9*).

Gas chromatograms (Fig. 1, *A* and *D*) of standard solutions showed that dieldrin and DDE have the same retention times on the columns used. Since we were interested in determining both the dieldrin and DDE in fat by gas chromatography, a preliminary separation of these compounds was required.

A column in which florisil is used has been described by McKinley *et al.*

Table 1. Concentrations of chlorinated hydrocarbon pesticides in the body fat of people in the United States, estimated by microcoulometric gas chromatography and by Schechter-Haller colorimetric analysis, and expressed as parts per million. (General population except where noted.)

Sample No.	Lipid (%)	Microcoulometric gas chromatography						Colorimetric analysis		
		BHC	Diel-drin	*p,p'*-DDE	*o,p'*-DDT	*p,p'*-DDT	Total as DDT	DDE	DDT	Total as DDT
		Samples collected in Wenatchee, Washington								
1	88	0.15	0.14	2.35	0.63	0.50	3.74	3.7	2.5	6.6
2	68	0.15	0.27	2.00	<*el**	0.86	3.09	4.0	1.9	6.3
3	75	0.11	0.16	10.00	5.00	1.25	17.39	17.8	8.1	27.9
4	66	<*el*	0.25	1.72	<*el*	0.73	2.65	4.0	2.0	6.4
5	58	0.70	<*el*	3.38	<*el*	1.04	4.80	6.7	2.9	10.4
6	60	0.50	0.36	3.80	<*el*	1.18	5.41	4.9	1.9	7.3
7	46	<*el*	0.23	1.86	0.92	0.73	3.72	3.8	2.3	6.5
8	62	<*el*	0.23	5.14	2.20	1.63	9.55	3.8	2.7	6.9
9	50	<*el*	0.20	2.76	<*el*	1.66	4.73	6.9	4.1	11.8
10	70	0.27	0.32	10.40	2.43	3.25	17.27	8.6	4.8	14.4
11		<*el*	<*el*	0.76	<*el*	<*el*	0.85	2.1	0.7	3.0
		Samples collected in Phoenix, Arizona								
12	70	0.67	0.27	6.52	3.44	2.93	13.63	6.9	6.4	14.1
13	75	<*el*	0.34	9.07	2.27	4.97	17.34	9.7	9.8	20.6
14	74	<*el*	0.18	1.83	1.51	0.68	4.23	5.5	2.5	8.6
15	74	0.69	0.18	3.38	1.51	1.16	6.43	2.8	1.1	4.2
16	52	0.20	0.09	3.17	0.88	1.16	5.57	4.5	2.3	7.3
		Samples collected in Louisville, Kentucky								
17	76	0.33	0.07	8.94	3.40	3.06	16.42	22.4	10.7	35.6
18	42	<*el*	<*el*	0.69	<*el*	<*el*	0.77	4.1	0.4	5.0
19	63	0.14	0.11	1.34	<*el*	0.48	1.97	2.1	0.9	3.2
20	60	0.22	0.14	3.04	1.12	1.13	5.64	4.4	3.2	8.1
21	86	0.24	0.09	1.73	<*el*	0.47	2.40	5.2	3.9	9.7
22		0.19	0.16	1.94	<*el*	0.70	2.86	5.8	3.3	9.8
23		0.41	0.04	6.00	<*el*	1.79	8.47	11.7	9.1	22.1
24	42	<*el*	<*el*	6.76	8.24	<*el*	15.77	12.5	11.0	24.9
25	65	<*el*	<*el*	5.73	0.95	0.98	8.31	4.5	3.6	8.6
26	58	0.09	0.02	0.48	0.35	0.23	1.11	1.5	0.9	2.6
27	67	<*el*	<*el*	0.55	<*el*	<*el*	0.61	2.1	1.2	3.5
28		0.20	0.11	2.10	0.44	0.52	3.30	2.8	1.3	4.4
		Samples from people who had experienced occupational exposure to pesticides								
29†	72	0.36	0.27	11.80†	2.71†	2.54†	18.39†	15.2†	12.1†	29.0†
30‡	72	0.47	0.36‡	3.28	2.43	<*el*	6.08	6.0	2.0	10.8
		Mean ± standard error§*								
		0.2 ±0.04	0.15 ±0.02	3.82 ±0.54	1.30 ±0.35	1.14 ±0.21	6.69 ±1.02	6.23 ±0.88	3.71 ±0.55	10.71 ±1.51

* Experimental limits (*el*) by gas chromatography at 128 ohm, 5 mv: BHC, 0.03 ppm; dieldrin, 0.02 ppm; *p,p'*-DDE, 0.06 ppm; *o,p'*-DDT, 0.09 ppm; *p,p'*-DDT, 0.09 ppm. † Applicator of DDT more than one year before sample taken. ‡ Applicator of aldrin within last year. § Means and standard errors exclude values for people who experienced occupational exposure.

(*10*) for the separation of DDT, DDE, and DDD [1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethane] from dieldrin, methoxychlor [1,1,1-trichloro-2,2-bis(*p*-methoxyphenyl) ethane], and Kelthane [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethanol]. A similar column was used in the present work. Florisil, 60/100 mesh, was used, reactivated at 210°C for 3 hours in a vacuum oven. A column with an inner diameter of 19 mm, and measuring 250 mm in length, was packed dry with 14 cm of florisil. About 1.5 cm of anhydrous sodium sulfate was then added and the column was packed by gentle tapping of the side walls. Glass wool was used to retain the column pack. Enough benzene wetted the column to ensure uptake. Samples dissolved in benzene were added to the column. It was found that benzene hexachloride (BHC), DDT, DDE, DDD, aldrin, heptachlor, and heptachlor epoxide could be eluted quantitatively (95 to 100 percent) in the first 100 ml of benzene. Each column was further eluted with 50 ml more of benzene, and this eluate was found to contain no pesticide. This gave assurance of complete separation of these materials from dieldrin and methoxychlor, which were finally eluted quantitatively (99 to 100 percent) from the column with 100 ml of 1 percent acetonitrile in acetone.

The benzene eluates of fat samples from this column were suitable for analysis. However, the acetonitrile-acetone eluates containing dieldrin required further cleaning. This was accomplished by an alkaline saponification followed by elution of the saponification products that were soluble in *n*-hexane, through an alumina-packed column with ether-hexane as described by Cueto (*11*).

Samples of each of the clean benzene eluates and the ether-hexane eluates were then separately subjected to microcoulometric gas chromatography (*7*); the samples of the benzene eluates were also analyzed by the Schechter-Haller method. To confirm the identity of materials present in fat, qualitative results were obtained from two different columns and under different operating conditions (Fig. 1). Benzene hexachloride (peak 1) was present in 19 of 30 samples; *p,p'*-DDE (peak 3 in chromatograms *B* and *E*) was present in all samples. Dieldrin (peak 3 in chromatograms *C* and *F*) was found in 25 of the 30 samples. Peak 4, which principally represents *o,p'*-DDT as no *p,p'*-DDD was found by paper chromatography (*12*), appeared in 18 of the 30 samples; *p,p'*-DDT (peak 5) was present in 26 of 30 samples. Quantitative results obtained by gas chromatography are presented in Table 1.

The mean concentration of dieldrin (0.15 ± 0.02 ppm) reported in this paper is in good agreement with that reported (*4*) for southern England. The mean concentration of BHC (0.20 ± 0.04 ppm) is considerably lower than the 1.19 ppm reported (*5*) for the French. The mean concentrations of *p,p'*-DDE, *o,p'*-DDT and *p,p'*-DDT in individuals who had not knowingly been exposed to DDT were 3.82 ppm, 1.30 ppm, and 1.14 ppm, respectively. There was no significant difference in the storage of BHC ($p = > .1$) or DDT ($p = > .5$) based on geographic source; however, the samples from Louisville, Kentucky, and nearby places contained significantly less dieldrin ($p = < .005$) than those from Phoenix, Arizona. The man with recent occupational exposure to dieldrin stored no more than one other subject who had not, to his knowledge, been specially exposed to it. The man who experienced occupational exposure to DDT more than a year earlier showed storage near the top of the range of the general population.

It was found that the ratio of *o,p'* to *p,p'* isomers of DDT stored in fat is not the same as their ratio in technical grade DDT. Since the Schechter-Haller method (with or without modification) was used in previous analyses of human fat for DDT-derived materials, there was no way of measuring the storage ratio of the isomers, but this ratio influences quantitative results obtained by the method. By necessity, the assumption that the *o,p'* and *p,p'* isomers of DDT would be stored in the fat in the proportion they occur in technical DDT (20 : 80) (*1*) was used in developing a two-color equation for the determination of *p,p'*-DDE and technical DDT (*6*). Among fat samples from 30 people, we found more of the *o,p'*-DDT isomer in 10 of the 16 individuals showing both isomers in their fat and in two others who showed no *p,p'* isomer, with an average of 1.54 times as much *o,p'*-DDT as *p,p'*DDT in the 18 people and 1.31 times as much in the entire group.

Standards were run by Schechter-Haller analysis in which the ratios of *o,p'*- to *p,p'*-DDT were varied while holding DDE constant and then applying the two-color equation for technical DDT and *p,p'*-DDE. When the ratios of *o,p'*- to *p,p'*-DDT were 20 : 80, 50 : 50, and 80 : 20, respectively, the recoveries of DDE and DDT were 97 and 109 percent, 118 and 94 percent, and 131 and 72 percent, respectively. These recoveries were obtained when the amount of DDE was aproximately equal to the sum of the *o,p'*- and *p,p'*-DDT isomers. When DDE was decreased to one-half the sum of the *o,p'*- and *p,p'*-DDT isomers, and the isomer ratio was 80 : 20, the recoveries of DDE and DDT were 188 percent and 71 percent, respectively. This means that for fat samples in which *o,p'*-DDT is increased relative to *p,p'*-DDT (in comparison to technical grade DDT used in establishing the two-color equation), results obtained by the Schechter-Haller method reflect higher DDE and lower DDT than is actually present.

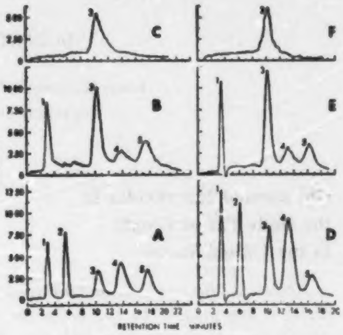

Fig. 1. Gas-coulometric chromatograms of a standard solution (*A* and *D*), and portions of a single extract of human fat (*B*, *C* and *E*, *F*) passed through two columns. First column (*A* to *C*): 1.22 m by 0.635 cm outer diameter, packed with 5 percent Dow oil grease on 30/60 mesh acid-washed Chromasorb P, at a column temperature of 182°C, nitrogen being used as a carrier gas at a flow rate of 88 cm³/min. Second column (*D* to *F*): aluminum, 1.83 m by 0.635 cm outer diameter, packed with 20 percent high-vacuum silicone grease on 30/60 mesh acid-washed Chromasorb P, at a column temperature of 215°C, nitrogen being used as a carrier gas at a flow rate of 240 cm³/min. The standard solution produced five peaks in chromatograms *A* and *D* representing: 1, benzene hexachloride; 2, aldrin; 3, dieldrin plus *p,p'*-DDE; 4, *o,p'*-DDT plus *p,p'*-DDD; and 5, *p,p'*-DDT. Chromatograms *B* and *E* were obtained from samples of the first 100 ml of benzene eluate from a florisil column, while chromatograms *C* and *F* were from samples of the final (acetone-acetonitrile) eluate from the same column. Peak No. 3 represents DDE in chromatograms *B* and *E* and dieldrin in chromatograms *C* and *F*.

The average total DDT-derived material expressed as DDT (6.69 ± 1.02 ppm) found by gas chromatography was only 62 percent of that found by the Schechter-Haller method for the same eluates (Table 1) and the difference is significant ($p = < .05$). Since recovery of standards by gas chromatography was good (95 to 100 percent), the differences obtained by the two methods when the same eluates were used must have been due to one or both of the following reasons: (i) Schechter-Haller calculation with equations based on a 20 : 80 storage ratio of *o,p′*- to *p,p′*-DDT, and (ii) variations in Schechter-Haller blanks.

By gas chromatography, DDE calculated as DDT averaged 62 percent of the total DDT-derived materials and agrees well with the 58 percent average reported for DDE by Hayes *et al.* (*2*).

Aldrin, heptachlor, heptachlor epoxide, and methoxychlor were not detected in any of the samples, but would have been detected had they been present in concentrations similar to those of BHC, dieldrin, and DDT-derived compounds.

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Determination of Chlorinated Insecticides in Human Blood

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The most important site of action of DDT, and perhaps other chlorinated insecticides, is the brain.[1,2] It has also been shown[3] that the concentration of DDT in blood tends to correspond to that in the brain. It is known that the concentration of a drug at receptor sites is related to the concentration of unbound drug in the plasma.[4] Brain samples are unavailable for most diagnostic studies; therefore, the concentration of chlorinated hydrocarbon insecticides in blood was studied, since it is readily available and of diagnostic value.

A number of methods have been developed for the determination of chlorinated insecticides in human blood utilizing gas chromatography and highly sensitive detectors.[5-7] All the methods, however, involved some cleanup procedure that was either destructive to some of the insecticides or was very time-consuming.

More recently, a rapid, simple, and sensitive procedure for the determination of hexane-extractable chlorinated insecticides in human blood involving no cleanup was developed in this laboratory.[8] Essentially, this method consisted of extracting 2 ml. of the sample with hexane, discarding the aqueous phase, drying the hexane with Na_2SO_4, and injecting an appropriate aliquot into a gas chromatograph equipped with an electron capture detector.

Studies of the efficiency of the method demonstrated in vitro binding of chlorinated hydrocarbon insecticides with some constituent in blood and strongly suggested the possibility of in vivo binding of these materials.

An equally simple, rapid, but more efficient method for the determination of chlorinated insecticides in blood has now been developed.

Method

Ten milliliters of blood was collected from each of 10 men and 10 women who had not been occupationally exposed to insecticides. The samples were placed in a 15-ml. ground glass stoppered centrifuge tube containing 0.1 ml. of 20% aqueous potassium oxalate as an anti-coagulant. The tubes were stoppered and the contents mixed gently by inversion. Three milliliters of oxalated whole blood were pipetted into another 15-ml. centrifuge tube, stoppered, and held for analysis. The remaining blood was centrifuged at 2000 rpm for 10 minutes, to separate the plasma from the red blood cells (RBC). After centrifugation the plasma was pipetted into another centrifuge tube.

Two-tenths milliliter samples of whole blood, plasma, and RBC were pipetted into test tubes fabricated from 7 mm. O.D. glass tubing cut in 6-inch lengths. After addition of the sample, the top of each tube was packed to a depth of 1½ inches with Analab's siliconized glasswool, to trap the more volatile insecticides. The tubes were then placed in an aluminum block (fig. 1) at 125°C. for 5 minutes. After removing the tubes from the block, the glasswool plugs were removed from the tubes and placed in small glass funnels that had previously been placed in 15 ml. centrifuge tubes. Nanograde hexane was added to each tube containing the sample. Also, a glass rod (2 mm.x 7 in.) was placed in the tube to aid in agitation of the sample during extraction on the mini-mixer. The samples were extracted three times with 1 ml. portions of n-hexane. The extracts were filtered quantitatively through the glasswool plugs into the centrifuge tubes. The funnels and glasswool plugs were then given a final rinsing with n-hexane. The n-hexane extracts were carried to dryness in a 40°C. waterbath with the aid of a gentle stream of clean

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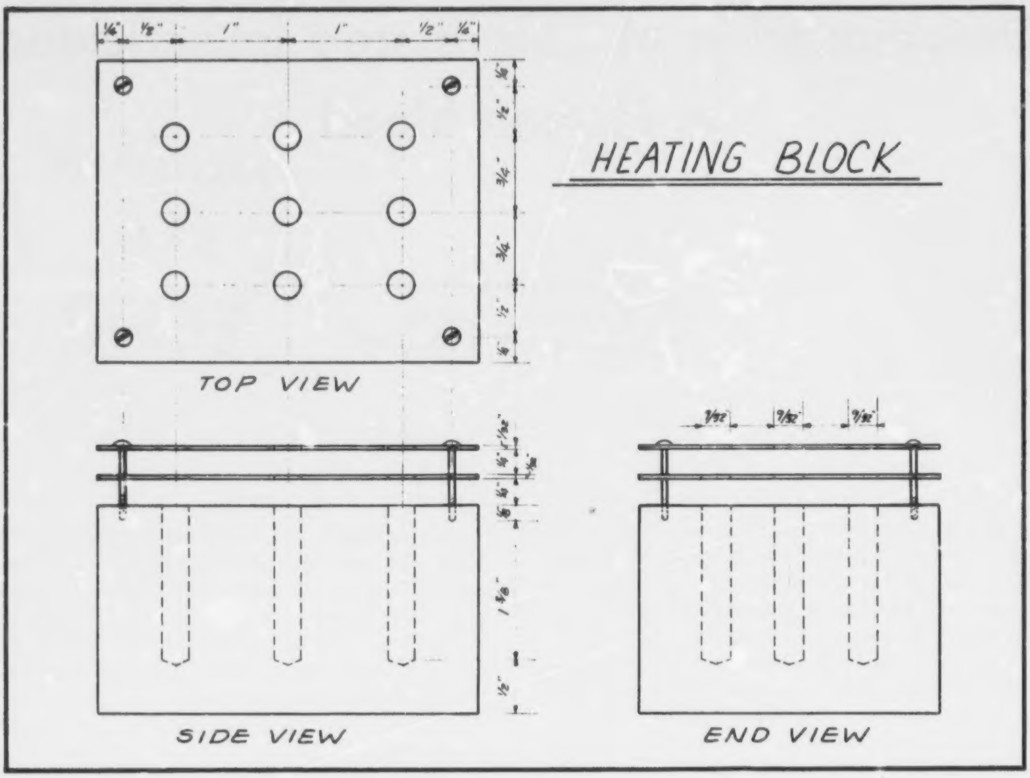

Figure I

dry air. The residues were taken up in n-hexane and an appropriate aliquot was subjected to gas chromatography.

A MicroTek MT-220 gas chromatograph equipped with a ^{63}Ni electron capture detector and a strip chart recorder was used. To confirm the identity of the materials in the effluent gas, two columns were used, both consisting of an aluminum tube 6 ft. x ¼ in. O.D. Column 1 was packed with 2.5% diethylene glycol succinate (DEGS) on 60/80 mesh, acid-washed chromosorb G. Column 2 was packed with 3% QF-1 on 60/80 mesh acid-washed chromosorb G. Both columns were operated under the following conditions: inlet and outlet blocks 224° C.; column oven 185° C.; detector ^{63}Ni, 290° C.; carrier gas N_2 60 ml./min. at 40 p.s.i.; power source, direct current 4 v. The retention times in minutes of columns 1 and 2, respectively, were as follows: p,p'-DDT, 24.0 and 33.8; o,p'-DDT, 12.8 and 22.4; p,p'-DDE, 9.2 and 17.6; o,p'-DDE, 7.2 and 13.4; p,p'-DDD, 30.5 and 31.2; alpha-BHC, 3.8 and 5.6; beta-BHC, 18.9 and 8.3; gamma-BHC, 5.9 and 7.0; delta-BHC, 17.1 and 9.2; heptachlor epoxide, 8.0 and 16.3; dieldrin, 11.9 and 25.4

Results and Discussion

To determine the efficiency of the method, 0.1 ml. of standard solution in n-hexane containing 10 to 165 times the amount of those materials usually found in 1 ml. of blood was added to each of 12 modified test tubes described above. This amount was used so that the concentrations present in water, whole blood, and plasma subsequently added could be ignored in the calculations. The hexane was allowed to evaporate to dryness. To four of these tubes was added 0.2 ml. of distilled water. To another group of four tubes was added 0.2 ml. of whole blood and, finally, 0.2 ml. of plasma was added to the remaining four tubes. The contents of each tube was mixed several times, using a mini-mixer, over a period of 2 hours. The samples were then treated as described in the method above.

Data presented in table I show that, in the presence of water, reasonably good recovery was obtained, ranging from 80% for beta-BHC to 100% for delta-BHC and p,p'-DDD, and all materials were volatilized and trapped in the tube above the level of the heating block. However, in the presence of whole blood or plasma, the recovery of added material was poor. Only one-

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third of the added insecticides was recovered from the upper portion of the tube. Therefore, extraction of the entire tube and its contents was decided on. Recovery from whole blood ranged from 14% for o,p'-DDT to 62% for alpha-BHC. Recoveries from plasma were essentially identical, ranging from 23% for o,p'-DDT to 60% for alpha-BHC. Recovery from whole blood and plasma of added materials was generally lower (table I) than that obtained by the simple hexane extraction method of Dale et al.[8]

TABLE I

Recovery of Chlorinated Insecticides and Related Materials Added to 1 ml. of Water, Whole Blood, and Plasma Comparison of Heat Vaporization vs. Hexane Extraction

Material	Micrograms Added	Heat Vaporization Water %	Heat Vaporization Whole Blood%	Heat Vaporization Plasma%	Hexane Extraction Plasma %
p,p'-DDT	0.2060	95	22	26	45
o,p'-DDT	0.2164	88	14	23	37
p,p'-DDE	0.2028	89	51	27	58
o,p'-DDE	0.2124	90	53	26	39
p,p'-DDD	0.1928	100	47	32	58
α-BHC	0.2096	93	62	60	85
β-BHC	0.2172	80	34	59	70
γ-BHC	0.2136	90	29	58	85
δ-BHC	0.1972	100	54	27	76
H.E.O.D.	0.1984	83	59	40	91
H.E.	0.1984	86	59	41	82

Eight plasma samples from four men and four women were then used to make a direct comparison between the heat volatilization method described above and the simple hexane extraction method for chlorinated insecticides and related materials. Significantly higher recovery of seven of the nine chlorinated hydrocarbon insecticides was obtained by the heat volatilization method than by the simple hexane extraction method (table II). The results obtained by the two methods for beta-BHC were not significantly different ($p > 0.05$). The heat volatilization method was inferior only for heptachlor epoxide, recognition of which was probably obscured by the increased amount of p,p'-DDE obtained by the volatilization method. Three compounds, o,p'-DDE, p,p'-DDD, and gamma-BHC, were detected quantitatively in blood of people from the general population for the first time by the heat volatilization method. Since standard materials were carried through the heat volatilization procedure without any apparent decomposition, the finding of measurable amounts of these additional materials can be attributed only to increased efficiency of the method.

Available knowledge does not permit prediction of the degree of extractability of chlorinated hydrocarbon insecticides present in blood through natural absorption and circulation. Therefore, the only fully independent and accurate way of testing recovery would be through the parallel chemical and radiometric studies of samples of blood from animals dosed with radioactive compounds. Since this radiometric method could not be used in man, we chose a technique for adding a known amount of compound to a sample that had proved to be the most severe test of recovery.

The results just discussed show that the bonding of chlorinated hydrocarbon insecticides in the test system and that in ordinary blood are not the same. This relation may be more common than is generally suspected, but this may be the first example in which the test was unrealistic by being too severe. No explanation of the nature of the difference can be given at this time.

The heat vaporization method was used to study the concentration and distribution of chlorinated insecticides and related materials in blood of 10 men and 10 women of the general population (table III).

If the hematocrit is considered to be 50%, these data show that the concentration of chlorinated hydrocarbon insecticides and related materials found in whole blood is in good agreement with the total concentrations found in plasma and RBC separately, i.e., consistent with the assumption that (plasma + RBC) /2 = whole blood.

Tables III and IV also show that men had significantly more of all of the chlorinated insecticides and related materials found in plasma

TABLE II

Comparison of Heat Vaporation Method with Simple Hexane Extraction Method for Determination of Chlorinated Insecticides in Plasma of People Not Occupationally Exposed.

Compound	Statistical Evaluation	Method of Analysis: Heat Volatilization	Method of Analysis: Simple Hexane Extraction	Significance
p,p'-DDT	Range Mean ± S.E.	0.0112-0.0492 0.0221 0.0044	0.0016-0.0053 0.0035 0.0004	$p < 0.001$
o,p'-DDT	Range Mean ± S.E.	0.0047-0.0211 0.0112 0.0018	0.0002-0.0065 0.0014 0.0007	$p < 0.001$
p,p'-DDE	Range Mean ± S.E.	0.0069-0.0291 0.01[illegible] 0.0026	0.0056-0.0092 0.0070 0.0004	$p < 0.025$
o,p'-DDE	Range Mean ± S.E.	0.0022-0.0065 0.0036 0.0005	<0.0001	
p,p'-DDD	Range Mean ± S.E.	0.0042-0.0248 0.0128 0.0023	<0.0001	
B-BHC	Range Mean ± S.E.	0.0036-0.0169 0.0069 0.0019	0.0016-0.0062 0.0031 0.0005	$p > 0.05$
G-BHC	Range Mean ± S. E.	0.0022-0.0129 0.0053 0.0011	<0.0001	
Dieldrin	Range Mean ± S.E.	0.0026-0.0066 0.0047 0.0005	0.0007-0.0023 0.0014 0.0001	$p < 0.001$
Heptachlor Epoxide	Range Mean ± S.E.	<0.0003	<0.0001-0.0013 0.0006 0.0001	

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than did women. A similar relationship was first reported by Robinson et al[9] for total equivalent-DDT in biopsy fat. Zavon et al[10] reported that dieldrin, DDE, p,p'-DDT, o,p'-DDT, and heptachlor epoxide were stored at a higher level in the fat of men than in the fat of women. In the RBC, the only compound in which there appeared to be a significant difference in concentration due to sex was o,p'-DDT ($p<0.05$) which was significantly higher in men. In the whole blood, the concentrations of insecticides were generally greater in men than women, but significantly different only for p,p'-DDE ($p<0.01$); p,p'-DDD ($p<0.025$) and dieldrin ($p<0.005$).

The concentrations of chlorinated insecticides in the plasma of men as compared with the concentrations in their RBC were higher in six of the eight insecticides detected in both the plasma and RBC, and the differences were significant for p,p'-DDE ($p<0.001$) and gamma-BHC ($p<0.025$) Although the concentrations of p,p'-DDD and dieldrin were lower in the plasma of men than in their RBC, the differences were not significant.

Five of the eight insecticides detected in both the plasma and whole blood of the men were greater in concentration in the plasma than in the whole blood, but were not significantly different. However, significantly greater concentrations of p,p'-DDD ($p<0.05$) and dieldrin ($p<0.05$) were found in the whole blood of men than in their plasma.

The concentrations of chlorinated hydrocarbon insecticides in the plasma of women as compared with the concentrations in their RBC were less in six of the eight insecticides that were detected in both the plasma and RBC, and the differences were significant for p,p'-DDD ($p<0.001$) and dieldrin ($p<0.001$).

Six of the eight insecticides detected in both the plasma and the whole blood of women were greater in concentration in the whole blood than in the plasma, and the differences were significant for p,p'-DDT ($p<0.05$); p,p'-DDD ($p<0.005$); gamma-BHC ($p<0.05$); and dieldrin ($p<0.05$).

These data show that p,p'-DDD and dieldrin are transported differently in men than other chlorinated insecticides, i.e., chifely in the red blood cells; also that women transport all of the pesticides differently than men in that they are transported equally well or better in the red blood cells of women.

The detection of heptachlor epoxide in the plasma of both men and women was probably obscured by the relatively large concentration of p,p'-DDE.

The method was applied to the serum of persons occupationally exposed to DDT from 10 to 19 years (average 15 years) during the manu-

TABLE III

Concentration and Distribution of Chlorinated Insecticides and Related Materials in Blood of 10 Men and 10 Women of General Population as Determined by Electron-Capture Gas-Liquid Chromatography and Expressed in Parts per Million.

Insecticide	Statistical Values	Plasma		Red Blood Cells		Whole Blood	
		Males	Females	Males	Females	Males	Females
p,p'-DDT	Range	0.0114-0.0490	0.0024-0.0164	0.0030-0.0343	0.0023-0.0265	0.0068-0.0844	0.0057-0.0199
	Mean	0.0253	0.0086	0.0153	0.0136	0.0248	0.0131
	± S.E.	±0.0038	±0.0014	±0.0031	±0.0024	±0.0073	±0.0014
o,p'-DDT	Range	0.0014-0.0129	0.0014-0.0039	0.0025-0.0054	<0.0003-0.0037	0.0014-0.0336	0.0016-0.0065
	Mean	0.0060	0.0024	0.0044	0.0022	0.0082	0.0033
	± S.E.	±0.0015	±0.0005	±0.0003	±0.0004	±0.0030	±0.0004
p,p'-DDE	Range	0.0101-0.0416	0.0059-0.0344	0.0046-0.0138	0.0061-0.0151	0.0062-0.0367	0.0068-0.0107
	Mean	0.0257	0.0135	0.0090	0.0091	0.0178	0.0088
	± S.E.	±0.0040	±0.0035	±0.0009	±0.0009	±0.0030	±0.0004
o,p'-DDE	Range	<0.0006-0.0096	0.0006-0.0023	<0.0007-0.0031	<0.0003-0.0039	<0.0003-0.0085	<0.0003-0.0025
	Mean	0.0036	0.0012	0.0012	0.0016	0.0022	0.0010
	± S.E.	±0.0011	±0.0002	±0.0003	±0.0004	±0.0008	±0.0003
p,p'-DDD	Range	0.0029-0.0199	0.0015-0.0066	0.0018-0.0425	0.0078-0.0319	0.0057-0.0448	0.0032-0.0150
	Mean	0.0083	0.0037	0.0121	0.0139	0.0189	0.0079
	± S.E.	±0.0018	±0.0005	±0.0040	±0.0024	±0.0041	±0.0011
β-BHC	Range	0.0024-0.0184	0.0008-0.0057	0.0012-0.0133	0.0012-0.0054	0.0013-0.0208	<0.0003-0.0035
	Mean	0.0065	0.0025	0.0047	0.0026	0.0060	0.0022
	± S.E.	±0.0015	±0.0005	±0.0012	±0.0003	±0.0018	±0.0004
γ-BHC	Range	<0.0005-0.0132	<0.0003-0.0019	<0.0005-0.0023	0.0007-0.0019	<0.0005-0.0080	0.0007-0.0018
	Mean	0.0057	0.0008	0.0008	0.0012	0.0015	0.0014
	± S.E.	±0.0019	±0.0002	±0.0002	±0.0001	±0.0008	±0.0001
Dieldrin	Range	<0.0012-0.0063	<0.0012-0.0022	0.0021-0.0069	0.0014-0.0108	0.0010-0.0129	0.0006-0.0045
	Mean	0.0028	0.0009	0.0043	0.0049	0.0055	0.0018
	± S.E.	±0.0007	±0.0003	±0.0005	±0.0009	±0.0011	±0.0003
Heptachlor Epoxide	Range			<0.0006-0.0036	<0.0003-0.0026	<0.0003-0.0120	0.0005-0.0051
	Mean			0.0012	0.0008	0.0023	0.0022
	± S.E.			±0.0004	±0.0003	±0.0011	±0.0005

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TABLE IV

Significance of Differences in Concentration of Chlorinated Hydrocarbon Insecticides and Related Materials in Human Blood as Determined by Sex and Blood Fraction.
Each difference is expressed as a probability (p) value determined by the method of Snedecor.[11]

	Males vs Females			Plasma vs RBC		Plasma vs WB	
Insecticide	Plasma	RBC	WB	Males	Females	Males	Females
p,p'-DDT	<0.001*	>0.70	>0.1	>0.05	>0.10	>0.50	<0.05*
o,p'-DDT	<0.025*	<0.05*	>0.1	>0.20	>0.50	>0.50	>0.10
p,p'-DDE	<0.050*	>0.90	<0.01*	<0.001*	>0.20	>0.10	>0.10
o,p'-DDE	<0.050*	>0.50	>0.1	>0.05	>0.40	>0.20	>0.50
p,p'-DDD	<0.025*	>0.60	<0.025*	>0.20	<0.001*	<0.05*	<0.005*
β-BHC	<0.025*	>0.10	>0.5	>0.20	>0.50	>0.50	>0.50
γ-BHC	<0.025*	>0.90	>0.50	<0.025*	>0.10	>0.05	<0.05*
Dieldrin	<0.025*	>0.50	<0.005*	>0.05	<0.001*	<0.05*	<0.05*
Heptachlor Epoxide		>0.20	>0.5				

*A value <0.05 is considered significant.

facturing of DDT. Each employee was rated as high or medium-low exposure, according to the nature of his work. Table V shows that the concentrations of total DDT-derived materials calculated as DDT correspond to the extent of exposure.

TABLE V

Average Concentration of Total DDT-Derived Materials Calculated as DDT in Serum of Occupationally Exposed People

Exposure Rating	Persons Exposed	Total DDT-Derived Materials Calculated as DDT
High	20	0.7371
Medium-Low	15	0.3950

Summary

Both the simple hexane extraction and the heat volatilization method give good recovery of chlorinated hydrocarbon insecticides from water. In vitro binding of all these materials can be demonstrated by both analytical methods. This suggests the possibility of binding in vivo, but it is certain that the two kinds of binding are not identical. The volatilization method gives lower recovery of added material, but it gives higher recovery of the same materials present through metabolism in blood taken from the general population. In fact, the volatilization method detects three compounds — o,p'-DDE, p,p'-DDD, and gamma-BHC — not detected by simple hexane extraction. Application of the volatilization method demonstrated higher concentrations of pesticides and their metabolites in the plasma of men as compared with the plasma of women, and the same was true in some instances of whole blood and RBC.

In general, men seem to transport chlorinated insecticides in their plasma at higher concentrations than in the RBC, the only exception being for that of DDD and dieldrin. Conversely, women transport chlorinated insecticides at higher concentrations in the RBC, and the differences are significant for DDD and dieldrin. Significantly greater concentrations of DDT, DDD, gamma-BHC and dieldrin were found in the whole blood of women than in their plasma.

The heat volatilization method was applied to unexposed persons and also to occupationally exposed people and the method follows the pattern of exposure.

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IMS **Toxicology**

Effect of Repeated Administration of Phenobarbital on the Metabolism of Dieldrin

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Since the detoxification of drugs and other foreign organic compounds by liver microsomal enzymes was first described by Brodie and his colleagues in 1955,[2] numerous papers have appeared on this subject. Conney and his coworkers,[3] who have been major contributors in this area of biochemical pharmacology, reported in 1960 on an increased activity of hepatic microsomal, drug-metabolizing enzymes induced by phenobarbital and other drugs. Hart and Fouts in 1963[4] presented evidence that the administration of certain chlorinated pesticides such as chlordane or DDT to rats produces a stimulatory effect on the metabolism of drugs. Recently, Street[5] reported a DDT antagonism to dieldrin storage in adipose tissue of rats. Thus, there exists evidence of the possible effects of pesticides not only on the metabolism of drugs but also on the metabolism of other pesticides.[6]

The reverse situation, that is the effects of drug stimulation of microsomal enzymes on the metabolism or storage of pesticides, has received limited attention. This is particularly true with reference to studies involving prolonged administration. However, Koransky *et al*[7] reported that a single dose of phenobarbital caused a marked acceleration of elimination of BHC following a single dose of alpha or gamma isomer. Street and his co-workers [8,9] studied the action of a ten-day dosage of aminopyrine, tolbutamide, phenylbutazone, and heptabarbital separately on the storage of dieldrin in the fat of rats.

The data presented in this paper show the influence of prolonged administration of phenobarbital on the storage of dieldrin.

Method

Studies were conducted on the effect of pretreatment with phenobarbital, as well as on its repeated administration concurrently with dieldrin. Three groups of 15 female rats were used. Rats in two of these groups were pretreated daily for seven days with intraperitoneal injections of phenobarbital (50 mg/kg). The barbiturate was then withdrawn from one of the groups, while the other was continued on daily phenobarbital administration. The third group was pretreated for the same period of time with intraperitoneal injections of normal saline solution and continued on this treatment throughout the experiment. After the pretreatment period, all rats were placed on the insecticide at a dosage of 0.5 mg/kg/day by stomach tube for 16 weeks. The animals were weighed at the beginning of the experiment and again after 2, 4, 8, and 16 weeks of dieldrin administration. At the 2-, 8-, and 16-week intervals, five rats from each of the three groups were sacrificed, and specimens of fat and liver were obtained for analysis. Feces collected at the same intervals were also analyzed.

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All samples for analysis were ground with sodium sulfate and extracted with n-hexane. The extract was evaporated, and the residue submitted to an ethanol potassium hydroxide saponification for cleanup purposes. The saponified mixture was diluted with water and extracted with n-hexane. This extract was dried with sodium sulfate and evaporated just to dryness.[10,11] The residue was taken up in a small volume of n-hexane, and appropriate aliquots, without further cleanup, were taken for gas liquid chromatography using the microcoulometric titrimeter for detection of chlorides.[12]

The samples were chromatographed on an aluminum column (3/16" x 10′) packed with 60/80-mesh, acid-washed Chromosorb G containing 5% by weight of QF-1 as the liquid phase. The column was maintained at 185 C, and a nitrogen carrier gas flow rate of 100 ml/minute was used. The column effluent was oxidatively decomposed at 900 C. The microcoulometric titration was recorded using a 10 millivolt recorder and a chart speed of 0.5 inch/minute. An integrator was used to determine the area under each peak. The ohms setting of the coulometer and the conditions of operation were such that a minimum of 0.1 μg of dieldrin could be detected quantitatively.

Results

Figure 1 shows the effects of repeated administration of phenobarbital on the growth of dieldrin-treated female rats. Although the rats treated with barbiturate for 16 weeks showed a consistently higher weight than the animals in the other two groups, the difference was not statistically significant, at least as determined on the basis of individual points. None of the rats developed any characteristic symptoms of dieldrin intoxication. However, those treated with barbiturates went through the expected period of sedation.

Figure 2 shows the concentration of dieldrin in fat and liver with respect to duration of dosage with the insecticide. No significant difference in the fat storage of dieldrin was found between the animals receiving only a seven-day pretreatment of phenobarbital and those receiving no barbiturate at all. However, a significant decrease in storage was found at all times between the animals given concurrent doses of phenobarbital and dieldrin as compared with those receiving dieldrin only. It should be noted that after the eighth week of treatment with phenobarbital and dieldrin, there appears to be an increase in the rate of storage and that this rate then parallels that of the rats not receiving phenobarbital.

The lower graph of Figure 2 shows that the concentration of dieldrin in liver ws not significantly different in the three treatments, except at the eighth week of administration of dieldrin when significantly less storage was found in the animals given repeated doses of phenobarbital and dieldrin as compared with those receiving dieldrin only. As expected, the concentration of dieldrin in the liver was considerably lower than that found in the fat.

Analysis of feces for dieldrin revealed no significant difference among the levels of the pesticide excreted by the three groups of rats. Only a small percentage of the daily dosage administered was recovered in the feces, and no metabolites of dieldrin were detected by the method used.

Discussion

Although O'Donnell and co-workers[10] have shown that, for the analysis of dieldrin, fat may be subjected directly to an alcoholic saponification without prior extraction, it became evident early in our study that this was not the case for liver or feces samples. With each of these latter specimens, two derivatives of dieldrin as determined by gas chromatography were formed during direct saponification in the presence of liver or feces. Dieldrin added to control liver or feces was completely converted by direct saponification to two unidentified materials. These two degradation products were not found on saponification of dieldrin added to a n-hexane extract of liver or feces. For this reason, all samples analyzed were first extracted with n-hexane and then subjected to an alcoholic saponification for cleanup. The gas chromatograms, at the sensitivity used, yielded only the peaks of p,p′-DDE and of dieldrin. These same typical chromatograms were obtained in animals treated with dieldrin only or concurrently treated with phenobarbital and dieldrin. Thus, the effects of the barbiturate on metabolism and storage of dieldrin are not qualitative but are quantitative so far as present evidence is concerned.

Indications of highly water-soluble metabolic conjugates of dieldrin in rat bile but not in feces have been reported by Heath and Vandekar.[13] Morsdorf *et al*[14] reported the detection of similar water-soluble metabolites in the bile of rats and rabbits on administration of aldrin or dieldrin. Ludwig *et al*[15] fed aldrin to male rats and established the presence by paper chromatography of aldrin, dieldrin and a mixture of several water-soluble unidentified metabolites in both urine and feces. Although in our study no fecal metabolites of dieldrin were detected, any water-soluble, highly polar, or acidic metabolite would not have been extracted by hexane from the diluted alcoholic saponification mixture. Therefore, the possibility that highly polar metabolites of diel-

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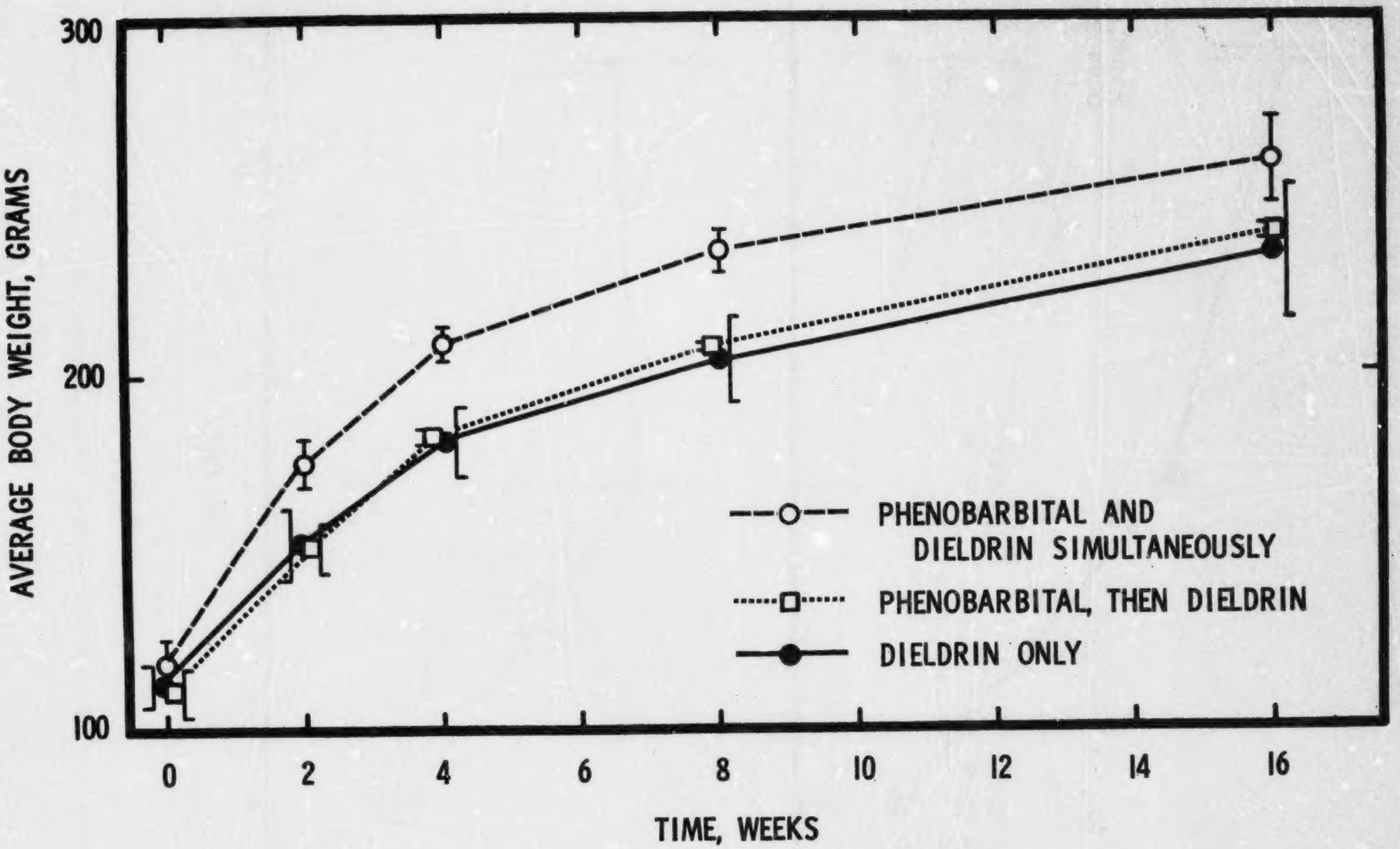

Figure 1

Body weight of female rats during dieldrin administration. Bars indicate standard errors. Rats were pretreated with phenobarbital (or with saline in the case of controls) for seven days just prior to the experimental period shown in the graph.

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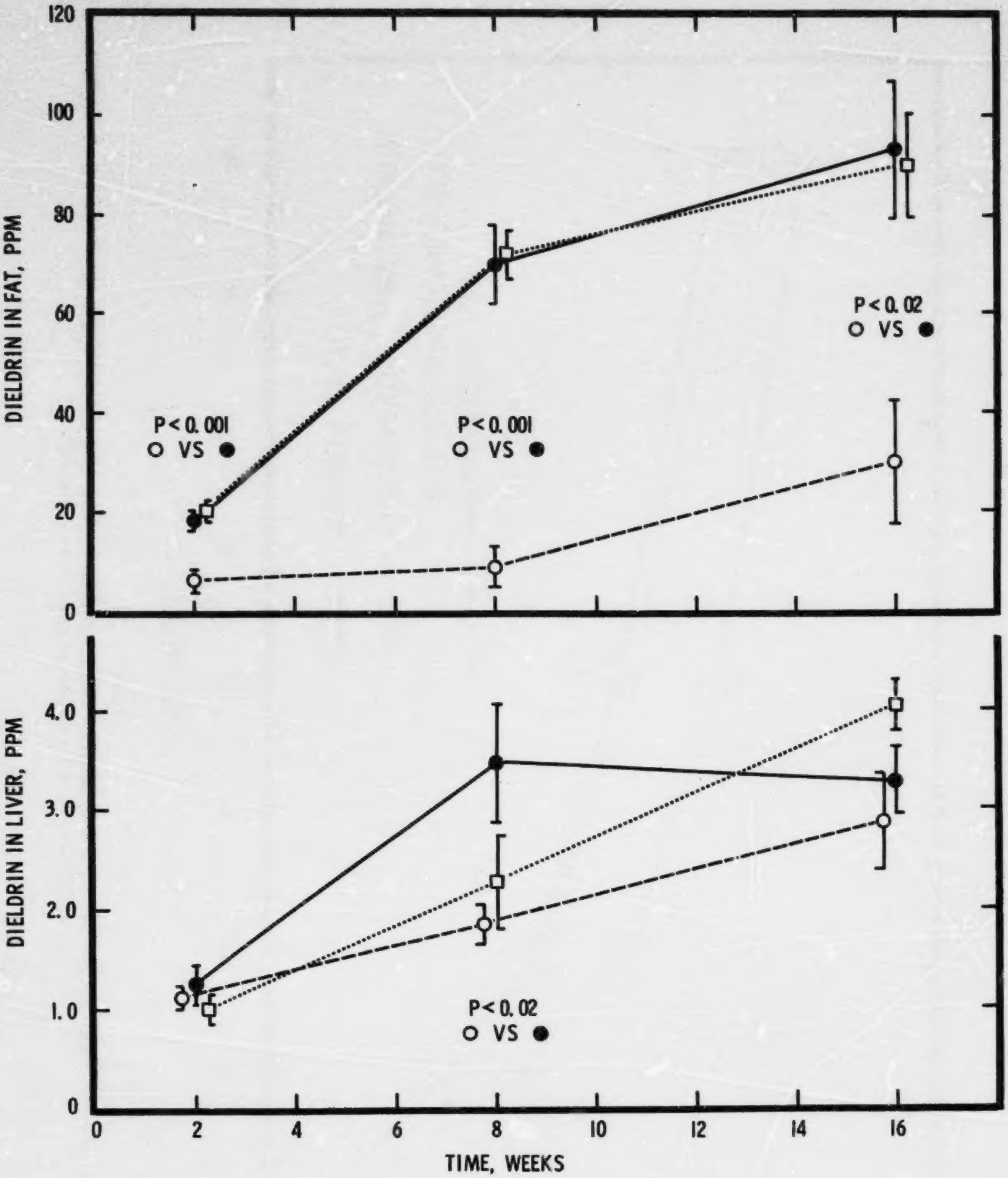

Figure 2

Storage of dieldrin in fat and liver of female rats during dieldrin administration. All conventions as in Figure 1.

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drin are excreted is not excluded. In fact, the data presented in this paper demonstrate an increased metabolism of dieldrin in phenobarbital-treated rats. It is evident that an analytical problem in the detection and determination of the excreted metabolites is involved.

Coulson and McCarthy[16] reported—and earlier, very preliminary results in this laboratory apparently confirmed—that rats fed dieldrin daily attain a maximal fat storage of the insecticide in about eight weeks, and thereafter the concentration decreases rapidly to a lower level in about eight additional weeks. The change in concentration of dieldrin stored in fat could not be accounted for by changes in eating habit or body weight. One of our original objectives in the present study was an attempt to explain this phenomenon by considering the possibility that dieldrin, on repeated administration, eventually stimulated its own metabolism to the extent that the concentration in fat decreased after reaching an initial peak. However, the data in Figure 2 for fat of rats on dieldrin only do not confirm these earlier observations; like those for fat, the levels of the insecticide in liver at 8 and 16 weeks of dieldrin administration are not significantly different. These data offer no evidence that dieldrin ultimately stimulates its own metabolism. On the other hand, Fouts,[17] using hexobarbital sleeping time as an index, demonstrated in mice that a single dose of dieldrin increased liver microsomal enzyme activity. Dieldrin may indeed stimulate its own metabolism, but further studies are needed to clarify this point.

The data presented in this paper show that phenobarbital decreases the storage level of dieldrin in fat without any appreciable effect on the concentration of the pesticide in liver. The decreased level in fat may be explained on the basis of an increased liver microsomal enzyme activity. As the metabolic turnover in the liver increases, more of the metabolized pesticide is excreted and less dieldrin is channeled to the fat depots. The rate of liver metabolism of dieldrin does not appear to be a limiting factor in the storage of dieldrin in the liver itself. It is safe to assume that, in contrast to fat, the presence of dieldrin in the liver is more dependent upon dosage and fatty content than upon rate of biotransformation.

Summary

Suitable doses of phenobarbital decrease the rate of storage of dieldrin in fat, but have little effect on the level of dieldrin in liver.

The authors acknowledge the technical assistance of Mrs. Barbara Adam, Messrs. Virlyn W. Burse, M. Frank Copeland, Thomas B. Gaines, and Mrs. Katharine L. Schlag.

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PESTICIDE MORBIDITY IN SOUTH CAROLINA

PESTICIDE
PUBLICATION
C 3 1
Food and Drug Administration
Chamblee, Georgia 30341

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R. H. GADSDEN, Ph.D.‡

Pesticide morbidity for South Carolina has not been previously recorded. Pietsch, et al[1] in gathering morbidity information from the State's Poison Control Centers reported that about 600 cases of all type poisonings occurred annually, about 10 per cent of these being pesticide poisoning.

With the use of pesticides increasing,[2] along with public concern about their usage, it is desirable that more accurate pesticide illness information be obtained. Indeed, if pesticide intoxications are rampant, it would be the obligation of the medical community to learn more of diagnostic techniques and tools as well as treatment procedures.

Methods

One thousand one hundred and fifty-seven South Carolina practicing physicians were queried by letter about the number of pesticide poisoning cases they had seen or treated in the previous year. A stamped postal card was enclosed for convenience of reply with the following information requested:

> Number of pesticide poisoning cases seen or treated in the last 12 months.

Table 1

Pesticide Poisoning Cases by Counties

County	No. of Cases Reported	County	No. of Cases Reported
Abbeville	1	Greenwood	6
Aiken	2	Hampton	1
Allendale	20	Horry	54
Anderson	9	Jasper	0
Bamberg	5	Kershaw	11
Barnwell	1	Lancaster	2
Beaufort	11	Laurens	4
Berkeley	3	Lee	6
Calhoun	10	Lexington	22
Charleston	12	Marion	19
Cherokee	10	Marlboro	5
Chester	2	McCormick	0
Chesterfield	5	Newberry	0
Clarendon	10	Oconee	23
Colleton	5	Orangeburg	30
Darlington	22	Pickens	2
Dillon	14	Richland	26
Dorchester	4	Saluda	2
Edgefeld	8	Spartanburg	21
Fairfield	12	Sumter	4
Florence	54	Union	1
Georgetown	1	Williamsburg	12
Greenville	72	York	28

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Opinion about pesticide poisonings, whether increasing, decreasing, or staying about the same.

While other information was desired it was thought that additional requests would diminish response.

Results and Discussion

Of 1157 practicing physicians queried, 667 reported they had treated or seen 572 cases of pesticide poisoning in the 12 months ending July, 1969. Most physicians reported that they believed the number of pesticide poisonings was about the same during the past 12 months as in previous years.

Most of the poisonings were concentrated in principal tobacco, cotton and peach growing counties. Greenville led the list of poisonings, reporting 72; Horry and Florence Counties ranked second, each having 54 cases. Table 1 reports the number of cases of poisoning by counties and Figure 1 presents a summary of this data by congressional districts.

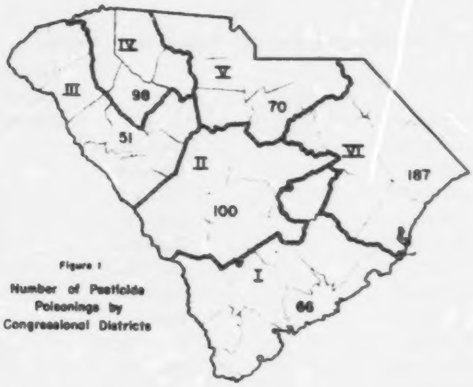

Figure 1

The morbidity data parallels Pietsch's[1] mortality report in that most poisonings occurred in the Sixth Congressional District. Horry County of this District had the most deaths in Pietsch's survey, which situation compared with that county's second rank in this morbidity study.

Table 2

Pesticide Poisonings Per 100,000 Population by Congressional Districts

District	Rate Per 100,000
I	.16
II	.19
III	.16
IV	.22
V	.26
VI	.47
All South Carolina	.25

Table 2 lists pesticide poisonings per 100,000 population by congressional districts, and shows the Sixth District as having a rate of 0.47, almost twice that of the next highest district of the state as a whole. The state rate of 0.25 indicates the same disease incidence as hepatitis but less than rubella.

The relatively high rate for Congressional District VI may in part be explained by the intensive use of agricultural pesticides in this tobacco and cotton area.

Summary

Of 1157 South Carolina physicians queried, 667 reported they had seen 572 cases of pesticides poisoning during the year ending July, 1969. One third of the cases, with a morbidity rate double that of the state, occurred in the Sixth Congressional District, an area characterized by intensive tobacco and cotton farming.

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Analytical Diagnosis of Pesticide Poisoning

PESTICIDE
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Environmental Protection Agency
Chamblee, Georgia 30341

Collection, Storage, and Shipment of Biological Samples

Glenn Morrison, MD, and William F. Durham, PhD

Approximately 150 deaths a year in the United States are attributable to pesticide poisoning, with total morbidity reaching up to 100 times that number.[1] Several sophisticated methods of analyzing biological samples for pesticides and their metabolic products have been developed in recent years. However, knowledge of the existence of these methods, of the sampling techniques required, and of the location of laboratories where the analyses may be performed has not been made generally available to the physician. The purpose of this communication is to bridge this gap so that the practicing physician can derive the most meaningful information from the analytical chemist.

General Considerations

First, the diagnosis must be made. Since several reports have dealt with various aspects of the diagnosis and treatment of acute pesticide intoxication,[2-5] these will not be covered here. Of preeminence is the maintenance of a high index of suspicion.

Once the diagnosis is entertained, and preferably before treatment has begun, samples for analytical determinations should be collected. The sophistication of a given analytical method is of no value if the sample to be analyzed has not been properly collected, stored, and shipped to the chemist. A good rule is to collect and save everything, including a sample of the suspected offending agent with its label, if available.

In general, all collecting and storing equipment should be glass and not plastic. Elements leached from the plastic may give extraneous and at times confusing peaks in gas-liquid chromatography or the pesticide may "coat" the plastic and thereby not be detected. Ideally, all stoppers should be of a screw-on metal type lined with aluminum foil previously washed with an organic solvent; plastic, rubber, and cork stoppers are to be avoided, although the siliconized rubber stoppers have not caused any difficulty.

If there is any doubt regarding immediate preservation or storage, the sample should be frozen. This is particularly advantageous if the nature of the toxicant is unknown and a preservative might interfere with the analysis.

Arrangements for transportation and analysis should be made as quickly as possible. If no facility exists nearby, one of the laboratories mentioned later in this communication may be contacted. All analyses should be carried out as expeditiously as possible.

If the samples are to be shipped any appreciable distance, they must be placed in adequate containers that are strong and well insulated. Various synthetic plastics (eg, expanded polystyrene foam, urethane) are probably the best insulators and heavy corrugated cardboard supplies an adequate support. The sample must be wrapped (eg, in foam rubber or corrugated packing material) to prevent breakage.

If freezing is required, the sample should be shipped in dry ice. If refrigeration will suffice, then the sample may be carefully surrounded by plastic bags filled with ice chips of various "refreezants" (water or silica gel packaged in plastic or metal and previously frozen).[6] All containers must be adequately labeled.

If various samples are being sent and cross contamination (eg, vapor) is a possible danger, then they should be shipped in separate containers. This is of particular importance if the samples vary greatly in their degree of contamination. For example, samples of the possible offending agent or clothing should not be shipped with biological samples.

Body Fluids

Blood.—Blood may be analyzed

From the Perrine Primate Research Branch, Division of Pesticide Chemistry and Toxicology, Bureau of Foods, Pesticides, and Product Safety, Food and Drug Administration, Perrine, Fla.

Reprint requests to Perrine Primate Research Branch, Division of Pesticide Chemistry and Toxicology, Food and Drug Administration, PO Box 490, Perrine, Fla 33157 (Dr. Morrison).

Determination of Pesticide Exposure

Biological Sample (In Glass Container)	Collection and Storage	Assays for Organochlorine (Example, DDT)	Assays for Organophosphate (Example, Parathion)	Assays for Carbamates (Example, Carbaryl)
Blood	10 ml, refrigerate or freeze	DDT, DDD, DDE*	Cholinesterase activity, perhaps parathion†	Cholinesterase activity†
Urine	100 ml, acidify and add 1 ml preservative or freeze	DDA	*p*-nitrophenol	1-naphthol
Gastric contents	Acidify and add preservative or freeze	DDT	Parathion	Carbaryl
Body tissue	200-500 mg (pea size), weigh and freeze	DDT, DDD, DDE	Parathion	...

*Any anticoagulant except heparin is suitable.
†Heparinized blood; separate cells and plasma.

directly for a pesticide or for a metabolic product, or indirectly for a biological effect of the toxicant. Heparin sodium is to be avoided if gas-liquid chromatography is utilized because it will often give extraneous peaks.

The chlorinated hydrocarbon insecticides, eg, DDT, may be assayed directly. Five to ten milliliters of venous blood (a minimum of 0.5 ml is required per assay) should be collected in any anticoagulant (preferably not heparin), the serum separated, and the sample kept cold or frozen, depending on the interval before analysis. However, recent evidence indicates that DDT will be degraded with time, even in frozen blood.[7] This degradation might be important in a monitoring study but probably would not be significant in an acute poisoning case.

More commonly in acute poisoning, an organophosphate insecticide will be the offending agent. These compounds are metabolized quite rapidly but their major biological effect, the inhibition of cholinesterase activity, can be measured. Many methods are available for measuring the serum and red blood cell cholinesterase (ChE) activity, including test tapes for use in the field.[8] Most laboratories use either the Michel electrometric procedure[9] or the pH Stat method.[10] For either of these methods, a sample of 5 to 10 ml of fresh venous blood is advantageous; however, the pH Stat method can be used with a minimum of 0.5 ml since only 0.2 ml of packed red blood cells or 0.15 ml of plasma is needed for analysis. Here heparin is the preferred anticoagulant because the other usual anticoagulants (oxalate, citrate, and edetic acid) will bind cofactors necessary for maximal cholinesterase enzyme activity.

Blood samples for ChE activity should be promptly centrifuged (eg, 2,000 rpm for 15 min) and the cells and plasma separated carefully so that the red blood cells are not hemolyzed. The cells should be washed once with saline solution and then each fraction refrigerated (2 to 4 C) or frozen (−20 C). The relationship of enzyme activity to storage time or temperature or both remains a moot question, but if the assay is done within a few days, either storage temperature will apparently suffice. However, red cells are hemolyzed by freezing, and if subfreezing storage temperatures are to be used, the cells must be completely separated from the saline wash solution or an accurate dilution (1:1) made.

Urine.—The analysis of pesticide metabolites in urine can often be valuable in making a diagnosis of overexposure or in following a patient after an acute intoxication. The most commonly detected chlorinated hydrocarbon metabolite is DDA, a metabolite of the class prototype, DDT. An overexposure to the organophosphates parathion, methyl parathion, and EPN would result in the urinary accumulation of the phosphate moiety and the metabolite *p*-nitrophenol. Current methods allow only for routine analysis of the latter compound. The third major pesticide metabolite that is generally detected in the urine is 1-naphthol, which is the principal urinary metabolite of the carbamate insecticide carbaryl (Sevin). In contrast with the organophosphates, carbamates are reversible inhibitors of cholinesterase and thus the measured blood cholinesterase activity may be higher (more normal) than would be predicted from the exposure. Therefore, the cholinesterase activity will not be a true reflection of the amount of exposure, but the urinary levels of 1-naphthol will be elevated.

Ideally, the urine should be collected in a wide-mouthed, screw-cap glass bottle. The bottle should have previously been cleaned with a chromic acid cleaning solution or its equivalent and then thoroughly washed with distilled water and sterilized.

Two methods of preservation are available. The first is to freeze the urine and ship it in dry ice. The second method is to add a preservative, eg, a few drops of 10% formaldehyde solution or toluene, and then acidify the sample. These steps are necessary because a basic medium (with ammonia formation) speeds the breakdown of any intact organophosphate while bacterial growth will cause the breakdown of *p*-nitrophenol to other nondetectable compounds. An acidified urine sample with added preservatives should be shipped cold, but it need not be frozen.

Although the analyses can be done with a few milliliters of urine, a 100-ml sample is easily collected and is preferable.

Methods for the analysis of these metabolites have been improving rapidly in recent years with the increasing use of the electron capture, gas-liquid chromatograph. New methods using this technique have recently been reported for DDA[11] and PNP.[12]

Other Fluids.—Gastric aspirates are primarily the other fluids of interest, although in rare circumstances the examination of the cerebral spinal fluid or fecal matter might prove helpful. The recovery and analysis of gastric contents in cases of oral ingestion is of paramount importance in making a diagnosis and assessing the severity of exposure. They are handled in same manner as the urine specimens, with freezing the method of choice for preservation.

Body Tissues

The last major area of biological sampling that is generally employed for pesticide exposure is the analysis of body tissues. The information derived from analyses of this type will depend on the particular pesticide in question.

The organophosphate and carbamate compounds are rapidly metabolized and therefore are not usually present as the intact molecule in body tissues. By contrast, not only are the chlorinated hydrocarbon compounds metabolized more slowly, but the parent compound and its metabolic products are stored in body tissues.[13] This storage takes place primarily in the body's lipid compartment and its sampling and analysis can yield much valuable information regarding chlorinated hydrocarbon exposure.

A limiting factor, aside from the particular pesticide in question, is the availability of body tissue. Fat samples can be readily obtained, eg, from the subcutaneous abdominal wall, but most other sites are less accessible.

In most cases 200 to 500 mg of tissue will be sufficient (ie, the size of a pea), although ten times this amount is easily obtained by biopsy and is required for confirmation. The tissue should be frozen quickly, or if only chlorinated hydrocarbons are to be considered, it may be preserved with 10% formaldehyde solution.[6]

If the sample is to be frozen, it should be weighed first and the weight recorded on the container as the freezing may distort the weight. In the case of postmortem material, large quantities (5 to 10 gm) of all tissues and organ contents should be saved. There is some evidence that, as with blood samples, the levels of DDT will decrease in tissue with time[14] so that analyses should be performed as quickly as possible.

Laboratory Services

The chemical tests discussed here are specialized in nature and with the exception of blood cholinesterase determinations, are not generally available in hospital laboratories. However, many state health departments, university laboratories, and commercial laboratories are equipped to carry out some of these analyses. The laboratories of the Division of Pesticide Chemistry and Toxicology, Bureau of Foods, Pesticides, and Product Safety, Food and Drug Administration, located in Atlanta, Ga, Perrine, Fla, Washington, DC, and Wenatchee, Wash, and the several contract Community Study Pesticide Projects of the Division of Community Studies of this agency[15] have the capability for carrying out these tests, and could be contacted for assistance or advice regarding human poisoning cases occurring within the states where they are located. Some states, such as California, have published a list of laboratories in their area which will run these tests.[16] In the event of doubt, physicians can contact their state health department or Poison Control Center for assistance in locating a laboratory to carry out the desired analyses.

The information in this article is summarized in the Table. By referring to this table, physicians confronted with possible pesticide poisonings will be able to select uniform methods of collection, storage, and shipment of samples.

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Behavioral and Physiological Effects of the Cholinesterase Inhibitor Carbaryl (1-Naphthyl Methylcarbamate)[1]

PUBLICATIONS
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Environmental Protection Agency

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SIDEROFF, S. I. AND J. A. SANTOLUCITO. *Behavioral and physiological effects of the cholinesterase inhibitor carbaryl* (1-*naphthyl methylcarbamate*). PHYSIOL. BEHAV. 9 (3) 459–462, 1972.—The anticholinesterase pesticide carbaryl was injected subcutaneously into rats lever-pressing for liquid reinforcement. During the test session a low intensity footshock accompanied each lever-press response with the carbaryl rats exhibiting a smaller response decrement due to the shock. In the second experiment, carbaryl injected rats displayed a higher behavioral threshold to footshock. These results appear to explain other studies in which animals given anticholinesterase agents show decreased efficiency in performing an avoidance response. Two additional experiments examined the effects of carbaryl on motivation for liquid. Rats lever pressing for liquid reinforcement on an FR-25 schedule were injected with carbaryl. It was found that the response onset after each reinforcement was significantly delayed in the carbaryl animals. This result, in addition to ad lib liquid consumption data, indicated a decreased drive for water in the carbaryl animals. ~~Our~~ findings, similar to results obtained using cholinolytics, presents an apparent discrepancy which ~~we~~ discuss in terms of (a) the necessity of having an optimum level of Acetylcholine (ACh) in the brain, (b) the possibility of a peripheral mechanism, or (c) the possibility of sympathomimetic effects of carbaryl.

Anticholinesterase Avoidance conditioning Carbaryl Footshock Pesticide Pharmacology Thirst

THE PESTICIDE carbaryl is a carbamate which produces cholinesterase inhibition [5]. Since its anticholinesterase activity occurs at doses lower than those causing signs of toxicity, discernible behavioral effects might also appear without symptoms of poisoning. A previous report by Giachetti, Chippi and Guerra [2] for example, found that low doses of another anticholinesterase, parathion, administered every other day for one month retarded the learning of a conditioned avoidance response. Similarly, introduction of an experimental carbamate, at doses well below toxic, levels considerably reduced the efficiency of rats in avoiding footshock [3].

We report here a series of experiments in which the effects of carbaryl administration on appetitive and aversive motivational properties in the rat are investigated. One objective of this study was to determine if the pesticide induced impairment of conditioned avoidance responses mentioned above was due to a learning or memory deficit or simply an increased tolerance to footshock. In addition we were interested in seeing if carbaryl instigated an increased rate of responding for liquid reinforcement, as would be expected from a cholinesterase inhibitor [4, 9].

In the first experiment, the rate of lever-press responding for liquid reinforcement was examined. In order to detect subtle differences in liquid motivation, a low-level footshock was introduced along with each lever-press. In the second experiment, thresholds to the footshock were examined in the control and carbaryl rats. In the third experiment we again looked for subtle differences in motivation for liquid by measuring the response onset latency after consumption was examined over a six week period with carbaryl injections given once a week.

EXPERIMENT 1

Method

Ten male Sprague–Dawley rats weighing between 200 and 250 g and approximately 60 days old, were used in Experiment 1. They were individually housed and placed on an ad lib food schedule. Each animal was water deprived for two days prior to bar-press training for water reinforcement.

Testing took place in a Lehigh Valley environmental cubicle and was controlled by electromechanical circuitry. Within the cubicle was a standard lever which, when depressed, activated a holding relay which permitted the lowering and raising of a water dipper with a capacity of 0.01 cc. The floor of the compartment consisted of a series of shock bars connected to a Lehigh Valley constant current stimulator and scrambler. Lever pressing was recorded by means of a Gerbrands cumulative recorder.

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[1] We thank Dr. N. Schneiderman for his helpful comments.

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The rats were trained to lever press for water on a continuous reinforcement schedule (CRF). Each session was 20 min long and was preceded by two days of water deprivation. Rats were allowed 30 min of free access to water prior to each deprivation period. After the rats were lever-pressing at a steady rate they were divided into experimental and control groups of 5 rats each. They were divided such that the groups' means were equal.

The 5 experimental animals were injected subcutaneously with a 1% solution of carbaryl in corn oil at 10 mg/kg (approximately 1/60 of the LD50). The 5 control rats were injected with corn oil. Preliminary investigations revealed that rats receiving this dosage of carbaryl exhibited a transient decrease in locomotor activity of 1–2 hr duration. Accordingly testing was always initiated two hours following injection. A 5 min baseline period of lever pressing for water was followed by a 20 min session during which each subsequent lever-press was accompanied by a 0.2 mA scrambled grid shock. Two test sessions were given, one week apart.

Results

The cumulative records of typical control and carbaryl injected subjects are shown in Fig. 1. During the baseline period the two animals display almost identical response rates. After onset of the contingent shock however, the control animal greatly reduced its rate of responding while the carbaryl animal showed a much smaller response decrement. The mean response rates of the 10 min shock period were 19.9% and 79.8% of the baseline for control and treated animals respectively. The difference was statistically significant ($t = 4.57$, $df = 18$, $p<0.01$).

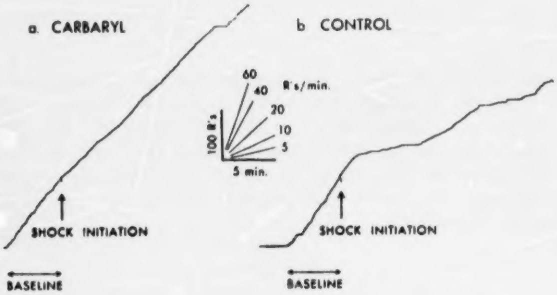

FIG. 1. Cummulative record of typical rats bar-pressing for water reinforcement on a CRF schedule. After 5 min baseline (at arrow), 0.2 mA footshock with each bar-press commenced.

EXPERIMENT 2

In this experiment, we were interested in whether the carbaryl group's performance of the previous test was due to a greater motivation for water, or to an increased tolerance to footshock.

Method

Ten naive male Sprague–Dawley rats, weighing 200–250 g were housed one to a cage and given food and water ad lib. The experimental enclosure consisted of metal walls and a clear Plexiglas top for observational purposes, and was 8.5 × 22.0 × 21.5 cm high. The floor consisted of shock-bars connected to a Nuclear-Chicago constant current stimulator.

Five experimental subjects were given 10 mg/kg of carbaryl subcutaneously in a 1% corn oil solution. Five control animals received just the corn oil. Two hr following injection each animal was placed in the enclosure and presented with a sequence of footshocks, first ascending and then descending in intensity. Shock was delivered only when the animal was motionless. Two observers, without knowing the treatment of the animals, independently assessed threshold intensity (as indicated by an "anticipatory" reaction or head turning response) and the intensity producing an aversive reaction (startle reaction or complete body movement). One week later the same procedure was repeated, i.e., the 5 treated and control animals were again injected with a carbaryl solution and oil respectively, and reactions of footshock were examined.

Results

The agreement between experimenters for scoring a stimulus-elicited "anticipatory" reaction and startle reaction, was 0.92 and 0.88 respectively. For both of these categories, carbaryl increased the response threshold. The mean stimulus intensities at which control and treated animals exhibited "anticipatory" reactions over the two testing sessions were 0.18 and 0.25 mA respectively. This difference was significant ($t = 2.84$, $df = 18$, $p<0.02$). The stimulus intensity necessary to produce a startle reaction for control and treated animals were 0.34 and 0.48 respectively. This difference was also significant ($t = 2.45$, $df = 18$, $p<0.05$). These results indicate an increased tolerance to shock in rats treated with carbaryl.

EXPERIMENT 3

The above experiment indicated an alteration in the effects of footshock on the carbaryl animals. While this was a probable cause of the results in Experiment 1, it was still possible that carbaryl was also affecting motivation for water.

Method

Sixteen male Sprague–Dawley rats weighing between 200–250 g and approximately 60 days old, were used in this experiment. The rats had free access to food and were placed on the water deprivation schedule used in Experiment 1. The apparatus used were also the same as in Experiment 1. The rats were trained to lever-press for water reinforcement on a CRF schedule. Once the animals were responding consistently, they were gradually raised to an FR-25 schedule. After a consistent lever-press rate was reached, each rat's mean time duration between each reinforcement and the subsequent onset of responding was calculated. The animals were matched on the basis of this response latency and divided into two groups. One group of 8 rats received subcutaneous injections of 10 mg/kg carbaryl in a 1% corn oil suspension. Eight control animals were given equivalent amounts of corn oil. In the subsequent test situation 2 hr after injection the average response latency was calculated over 80 reinforcements for each subject. The carbaryl subjects were then tested for 4 additional weeks. During this period, each rat received one injection of 10 mg/kg/week. They were tested 2 hr after each injection and again 48 hr later.

Results

The responding of 2 carbaryl rats broke down on the first injection day within the first 10 reinforcements. On this test day the average latency for the remaining 6 animals was 6.43 (±S.E. 0.86) sec as opposed to 3.75 (±S.E. 0.29) sec for the control group. This difference between treated and control group latencies was significant ($t = 3.31$, $df = 12$, $p < 0.01$). Examination of responding for the 6 carbaryl animals over the 4 additional treatment weeks revealed no significant change from the latencies on the first carbaryl day ($p < 0.02$).

EXPERIMENT 4

In this experiment we attempted to more clearly demonstrate the results of the previous experiment showing a decreased motivation for liquid by the carbaryl animals.

Method

Eighteen male Sprague–Dawley rats weighing between 200–250 g were housed one to a cage and allowed food and water ad lib. Body weight and water consumption were measured over a one-week period, at which time the rats were divided into 2 groups of 9 animals matched with respect to weight and water consumption. The carbaryl and the control groups were then injected once a week, for 5 weeks, as described in the previous experiments. The amount of water consumed by each rat was measured twice a week and its body weight taken just prior to dosing. Water bottles were fitted with ball-valve drinking tubes to minimize wastage. Water consumption was determined by weigh-back and expressed in grams.

Results

Figure 2 shows liquid consumption for the control and experimental groups over the baseline and treated weeks. It can be seen that the carbaryl animals showed a marked reduction in liquid intake. The mean water consumption of the first plus the last week and a half was obtained for each group. Statistical analysis comparing these means showed a significant difference ($t = 3.40$, $df = 106$, $p < 0.01$). The mean body weights of the two groups did not differ significantly over the five-week period ($p < 0.2$).

DISCUSSION

The results of Experiment 1, at first, indicated that rats injected with carbaryl when water deprived, have a greater drive for water than control animals under similar deprivation. In the first part of this experiment, in view of the results showing that both groups lever-pressed at similar rates, it was assumed that we were observing the upper limit of responding. In order to bring the baseline rate below this limit to observe any effects of carbaryl, the instantaneous, low level footshock was delivered with each lever-press. This, in effect, made the task more difficult and decreased the rate of responding for both groups; however, the control groups rate was significantly lower than the carbaryl groups. While the initial interpretation indicated an increased motivation for liquid produced by carbaryl, Experiment 2 yielded another possibility. In this experiment we found that both the "anticipatory" and the "startle" response thresholds in the rats were significantly higher in the carbaryl group. This increased tolerance to shock in subjects treated with carbaryl may account in part for the difference in the shock induced decrements between groups.

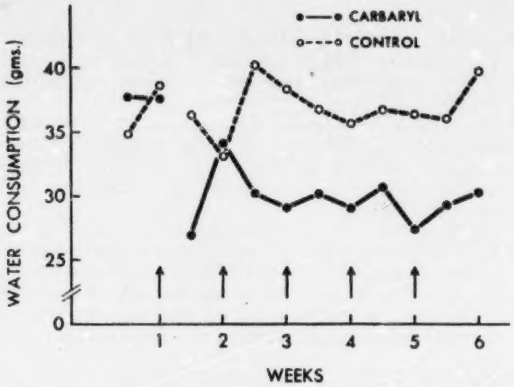

FIG. 2. Average daily water consumption in carbaryl and control groups, measured twice a week. The dosing is indicated by arrows.

While the evidence indicated the importance of the difference in sensitivity to shock, it was still conceivable that carbaryl as a cholinesterase inhibitor enhanced the animals' drive for water [9, 14]. In the third experiment, however, it was found that carbaryl injected animals consistently exhibited longer latencies in returning to the bar after receiving water reinforcement. Also, in the last experiment, the treated animals exhibited a decreased daily water intake. These results indicate that carbaryl reduces water consumption, thus suggesting that this pesticide influenced motivation in both appetitive and aversive situations.

The increased tolerance to shock induced by carbaryl in the present study affords a possible explanation for deficits in avoidance conditioning reported by Giachetti *et al.* [2] and Goldberg *et al.* [3]. Conceivably, in these experiments, there was less motivation to avoid or escape since the animals were less sentive to the footshock reinforcer. The result was less efficient performance. This would appear to be a more parsimonious explanation than any deficit in memory or learning.

These results are interesting in light of recent work indicating the same findings from introduction of cholinolytics [6, 10, 12]. These apparently contradictory results can be accounted for by an hypothesis put forth by Deutsch [1], whereby cholinergic pathways require an optimal concentration of acetylcholine to function properly. Thus, it is possible that too much or too little endogenous acetylcholine in central synaptic pathways could produce similar effects on the same system.

Interestingly, Khavari and Russell have published evidence that cholinolytic drugs including atropine and methyl atropine reduce water intake [7]. Since the latter drug does not pass through the blood brain barrier very effectively the authors indicate a probable peripheral action. Furthermore, they find decrements in drinking at dose levels which do not produce significant changes in central ACh levels. Thus, there might be a peripehral mechanism analagous to the central theory discussed above, in which an optimal peripheral level of ACh is required for normal functioning.

An alternative explanation is suggested by other observations in our laboratory which show that carbaryl has sympathomimetic properties on smooth muscle preparations *in vitro* [11]. If similar properties were found in the central nervous system it might support the contention of Leibowitz [8], that alpha adrenergic agonists activity suppress water consumption.

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PUBLICATION

7

TOXICOLOGY PROGRAM
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Relative Toxicity of Insecticides*

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and Wayland J. Hayes, Jr.[2]

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IT is of great importance that we achieve a balanced view of the problem which is before us. This may be done in part by reviewing certain statistics on fatal chemical poisoning. Table 1 reveals that chemical poisoning occupies a minor place as a cause of death. Accidents of a mechanical nature are more common. Table 2 shows that there has been no significant change in the rate of fatal chemical accidents during the last decade. This may be somewhat surprising when one considers the rapid increase in the number and tonnage of toxic compounds which has taken place during this same period. The quantity of a few pesticides produced in the United States is shown in table 3. Table 4 shows that deaths caused by arsenicals and a few of the other older pesticides, chosen for purposes of illustration, continue to occur at about the same rate as they did in 1940 before the introduction of the newer pesticides. This is explained by the fact that the newer toxicants have not so much supplanted as supplemented the older ones.

The relatively good safety record of the older economic poisons is due in part to the fact that workers are aware of their hazard and have learned to treat them with respect. Unfortunately, a part of their reputation for safety depends on a passive acceptance of a small but continuing series of accidents. This same kind of careless thinking may lead one to ignore the importance of common household chemicals and non-prescription drugs as a source of danger. Accidents associated with these materials are much more frequent than those caused by economic poisons. A home accident survey conducted by the Accident Prevention Service, District of Columbia Chapter, American Red Cross, has illustrated this over a period of years. Of 287 cases of fatal and nonfatal chemical poisoning in which a definite cause was listed in 144 instances, six were caused by insecticides and eleven by rodenticides, while at least 22 were ascribed directly to kerosene and 31 to aspirin. Another good illustration of the danger of household chemicals is the study by Rubin and his colleagues[4], who showed that among 250 consecutive admissions for the ingestion of poisons, 21 per cent of the children studied had taken kerosene, while only 9 per cent had taken one of a variety of pesticides.

The facts which have just been mentioned we believe, should be viewed as a challenge to the medical profession and the pesticide industry to reduce these preventable, and therefore unnecessary, accidents. The solution of the problem will, in the long run, be benefited by a balanced perspective. Scare tactics may create a flurry of intense activity and may result in the correction of real abuses. However, there is such a thing as crying "wolf" too often, and those who resort to scare tactics frequently may end by losing the sheep and the shepherd boy as well.

New Pesticide Accidents

A BRIEF review of the accidents associated with the newer pesticides may now be made. Thirteen fatal accidents have been clearly associated with sodium monofluoroacetate (1080) and five other deaths may have been caused by it. There have been at least six nonfatal cases. The established fatalities have varied from one to five per year since 1946. Al-

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* Paper presented at 38th annual meeting of C.S.M.A., Washington, D.C., Dec. 2, 1951

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[4] Clin. Proc. Children's Hosp. Washington, 5(3): 57-73, February, 1949.

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though figures are not available regarding production or sale of the compound, it is clear that the amount is small as compared with some of the other pesticides, including parathion. Furthermore, it is only because of the unusually strict controls voluntarily imposed by the manufacturer that the record has been kept as good as it is. It is possible that the introduction of warfarin, a relatively safe rodenticide, may lead to a more restricted use of 1080 and a continuation of the decrease in accidents caused by it—a decrease which seems to have been present for at least a year.

ANTU has a good safety record for man in spite of the fact that at least ten children are said to have eaten baits containing the rodenticide[5]. There remains a need for a fast-acting rodenticide that is safe for man and effective against all species of commensal rodents. Although the new slow-acting rodenticides have a good safety record, there remain rodent control problems which are more conveniently solved by a fast-acting rodenticide.

Among the newer insecticides, the organic phosphorus compounds have produced by far the most fatal accidents. Parathion has accounted for eight deaths in the United States, and there was one other in which the part played by parathion could not be evaluated clearly. A large number of nonfatal cases is known, and the majority is probably never reported. However, the very active educational campaign carried out by the manufacturers has done a great deal to bring information to the farmers and orchardists who buy and use the product. This campaign, along with the efforts of private, state, and federal agencies, has apparently reduced the accident rate, although use of parathion has increased. Industry is to be congratulated for its part in promoting safer practices in the use of economic poisons.

Tetraethylpyrophosphate (TEPP) has caused at least nine fatal accidents, and there are two other cases in which the diagnosis is not established. Among the chlorinated hydro-

TABLE 1

Deaths from selected groups of causes and crude death rates per 100,000 population. United States, 1948.[2]

Cause	*Number*	*Rate*
All causes	1,444,337	988.5
Infections and parasitic disease	73,016	50.0
Cancer and other tumors	202,386	138.5
Disease of the circulatory system	505,716	346.1
Chronic poisoning and intoxication	2,550	1.7
Alcoholism	2,433	1.7
Lead Poisoning	46	0.0
All other chronic poisoning	71	0.0
Violent or accidental deaths	123,009	84.2
Accidental deaths	98,001	67.1
Automobile accidents (except collisions with trains or street cars)	29,915	20.5
Accidental absorption of poisonous gas	2,002	1.4
Acute accidental poisoning by solids and liquids	1,436	1.0

[2] U. S. Public Health Service. Vital Statistics of the United States—Pt. I — 1939-48.

TABLE 2

Total accidental deaths in the United States caused by chemicals and rate per 100,000 population.[2]

	Gases		*Solids and Liquids*	
Year	*Number*	*Rate*	*Number*	*Rate*
1939	1,396	1.1	1,371	1.0
1940	1,562	1.2	1,324	1.0
1941	1,464	1.1	1,191	0.9
1942	1,694	1.3	1,193	0.9
1943	2,038	1.5	1,254	0.9
1944	1,907	1.4	1,381	1.0
1945	2,131	1.6	1,532	1.2
1946	1,874	1.3	1,536	1.1
1947	1,938	1.4	1,504	1.0
1948	2,002	1.4	1,436	1.0

[2] U. S. Public Health Service. Vital Statistics of the United States—Pt. I — 1939-48.

TABLE 3

Estimated production of selected pesticides in the United States, expressed in thousands of pounds.[3]

Year	*Calcium Arsenate*	*Lead Arsenate*	*DDT*	*BHC*
1939	41,349	59,569	—	—
1940	—	—	—	—
1941	56,136	75,912	—	—
1942	84,136	63,751	—	—
1943	74,854	73,955	—	—
1944	44,350	90,705	9,626	—
1945	25,644	70,522	33,243	—
1946	35,392	56,667	45,651	—
1947	46,904	31,006	49,600	8,197
1948	27,234	24,630	20,240	18,382
1949	16,006	16,866	37,904	27,937
1950	49,474	—	77,973	57,508

[3] Production statistics based on U.S. Tariff Commission and Bureau of Census figures.

TABLE 4

Number of accidental deaths in the United States for selected causes.[2]

Year	*Arsenic and Its Compounds*	*Nux Vomica and Strychnine*	*Tobacco and Derivatives*	*Creosol Compounds*	*Lye and Potash*	*Barbituric Acid and Derivatives*
1939	109	82	7	31	120	201
1940	73	67	7	32	111	246
1941	70	40	7	29	94	232
1942	71	50	12	25	87	197
1943	85	54	6	20	88	226
1944	77	54	12	17	74	270
1945	58	52	9	13	79	392
1946	63	45	11	19	90	436
1947	48	37	10	7	72	418
1948	63	30	8	8	74	419

[2] U. S. Public Health Service. Vital Statistics of the United States—Pt. I — 1939-48.

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[5] Emlen, J.: *Amer. J. Pub. Health*, 37(6): 721-727, June, 1947.

(2)

carbon insecticides, chlordane is known to have killed one factory worker. In a second case, the diagnosis is in doubt.

Some fourteen deaths have been listed as resulting from DDT intoxication. All but two of them involved ingestion and, in most, the action of the solvent in the formulation undoubtedly played an important if not deciding role. Two fatalities ascribed to DDT involved *agranulocytosis* and *periarteritis nodosa*. The diagnosis of DDT poisoning in these instances stands on the weakest of foundations.

It is, of course, recognized that there are usually several nonfatal accidents for every fatal one. The reporting of the fatal ones is much better, however, and they serve as a very good indicator of the seriousness of acute poisoning in general.

Chronic poisoning may, in specific instances, be much more significant than acute poisoning. Chronic intoxication is recognized in our vital statistics. As shown in table 1, approximately two thousand five hundred cases are reported annually. Alcoholism accounts for the greater proportion, while the remainder—about five per cent—includes poisoning by lead and a wide variety of other materials. At the present time, cases of chronic poisoning in man by the newer pesticides have not been confirmed unless one lists in this category certain cases involving occupational exposure to organic phosphorus insecticides. If one may judge from animal experiments, intoxication following repeated exposure to small doses of the newer pesticides would be similar to illness caused by a single large dose. Such a relationship does not hold for all chemicals; for example, chronic and acute lead poisoning may be quite dissimilar. We must, therefore, be on the alert for syndromes characteristic of chronic illness. Furthermore, we must remember that man, in addition to being able to report his symptoms, may show different signs of illness than are shown by laboratory animals.

In this connection, a word of caution is needed. While remaining on the alert for exceptions, we cannot afford to forget the general rules. Any case of human illness ascribed to poisoning should be accompanied by convincing evidence of excessive exposure and by evidence that the signs and symptoms resemble those known to be characteristic. If this cannot be done, a diagnosis of specific intoxication should not be made unless every effort has been made by competent specialists to rule out other causes. Careful attention should be given to the principles of epidemiology. In general, those persons with the greatest exposure to a compound will show the greatest effects—if effects are to be shown. This is true even of those materials, such as beryllium, in which there is good evidence that a sensitization reaction constitutes a significant part of the mode of action.

A word of caution is also in order regarding the expression "chronic toxicity." In simple terms, the expression means prolonged poisoning. It is generally caused by repeated small exposures to a toxicant but, in certain instances, it may be caused by one or a few large doses, as with arsenic. Conversely, small repeated doses may result in acute poisoning. An example is poisoning by small, repeated doses of parathion, in which the cholinesterase level is successively lowered until reaching a point where symptoms of acute poisoning occur.

In conclusion, the newer pesticides have, in comparison with the older ones, a good safety record. This is in large part due to intelligent enforcement of the Federal Insecticide, Rodenticide, and Fungicide Act, and of similar legislation in the various states. Educational campaigns carried on by a great variety of groups, including industry, have helped tremendously to familiarize users with the facts and to establish the custom of reading the labels on economic poisons. The American Medical Association, The U. S. Public Health Service, and others have called the attention of physicians to the symptomatology and treatment of intoxication caused by various pesticides.

Since a great many new pesticidal compounds have been introduced in the last decade, it is obvious that many problems require further scientific study. There is nothing to be gained in the long run, however, by irresponsible statements that nothing is now known of the toxicology of the newer pesticides, or that no legal control of their use exists, or—in the absence of epidemiological proof—that a wide variety of illness from which mankind has suffered for generations is now caused by intoxication by the newer economic poisons.

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DHEW-ATLANTA, GA.

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PESTICIDE
PUBLICATION

No. 640

Environmental Protection Agency
Chamblee, Georgia 30341

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Chlorodibenzo-*p*-dioxin Contamination of Two Commercially Available Pentachlorophenols

A comparison was made of chlorodibenzo-*p*-dioxin contamination in a technical and an analytical grade of commercially available pentachlorophenols. Hexa-, hepta-, and octachlorodibenzo-*p*-dioxin were found in both samples. The technical product was more contaminated than the analytical one by factors of 1400, 600, and 539 for the hexa-, hepta-, and octachlorodibenzo-*p*-dioxin, respectively. Tetrachlorodibenzo-*p*-dioxin was not found. Results were determined by gas chromatography and confirmed by infrared spectrophotometry and mass spectrometry. Mass spectral analysis indicated the presence of polychlorodibenzofurans and polychlorodiphenyl ethers. A distinction was made, using gas chromatography-mass spectrometry, between the chlorodibenzofurans observed as original constituents of the pentachlorophenol and those derived from the fragmentation of chlorodiphenyl ethers.

Recent research has shown that some chemicals receiving widespread use in the environment contain contaminants which are more toxic than the major compound (Goursaud *et al.*, 1972; Vos *et al.*, 1970; Woolson *et al.*, 1972). Studies should be conducted to determine the acute and chronic effects of the major compounds both with and without the toxic contaminants.

It is established that the conditions of manufacture for pentachlorophenol (PCP) can give rise to the toxic contaminants chlorodibenzo-*p*-dioxins (CDD's) (Higginbotham *et al.*, 1968; Jensen and Renberg, 1972). The toxicities of some of the compounds in this series have been reported (Higginbotham *et al.*, 1968; Rowe *et al.*, 1971; Williams *et al.*, 1972).

This paper reports an assessment of the levels of contamination of two commercially available PCP's: a technical product containing 86% PCP and a product of analytical grade containing 99+% PCP.

EXPERIMENTAL SECTION

The CDD's were extracted with hexane from an aqueous solution of the sodium pentachlorophenate and the extract was chromatographed on alumina columns according to the method of Firestone *et al.* (1972). Although four fractions were collected from alumina, only fractions III and IV were subjected to sulfuric acid cleanup (Firestone, 1971) and, subsequently, analyzed.

CDD concentrations in fractions III and IV were determined by gas chromatography (gc) coupled with electron capture (EC) ^{63}Ni, Coulson conductometric (CC), and flame ionization (FI) detectors. A Tracor 550 gas chromatograph was equipped with a ^{63}Ni EC detector and a 6 ft × ¼ in. o.d. glass column packed with 3% OV-1 on 80/100 mesh Supelcoport. The column temperature was 220°, and the nitrogen flow was 135 cm^3/min at 60 psi.

A Micro-Tek 2000 with a FI detector was used with a 6 ft × ¼ in. o.d. stainless steel column packed with 3% SE-30 on 100/120 mesh Chromosorb W, DMCS treated, AW. The column temperature was 220°. With a head pressure of 40 psi, the flow rates in cm^3/min are as follows: nitrogen, 80; oxygen, 31; air, 39; and hydrogen, 181.

A Micro-Tek 2000 was also used with a CC detector. A 6 ft × ¼ in. o.d. glass column was packed with 3% OV-1 on 70/80 mesh Chromosorb G, DMCS treated, AW. The column temperature was 220°. Helium flow was 65 cm^3/min at 40 psi.

Under these conditions, the limits of sensitivity for tetraCDD on EC, FI, and CC are 1.0, 10.0, and 2.0 ppb, respectively. Gas chromatograms were compared qualitatively and quantitatively to those of standard CDD solu-

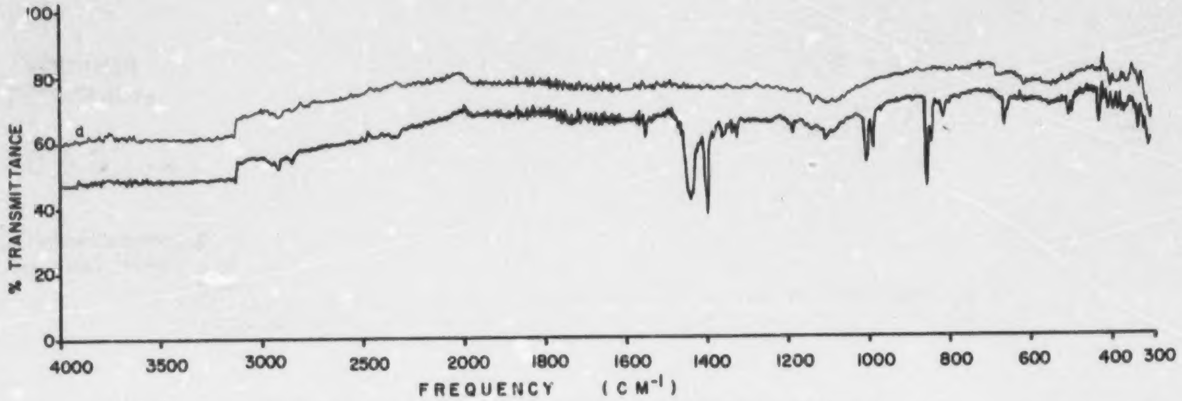

Figure 1. Infrared spectrum of a precipitate from fraction III of the technical PCP extract. a = blank.

Table I. Dioxin Concentration Found by gc Analysis in ppm

Sample	Detector	Hexa-CDD	Hepta-CDD	Octa-CDD
Technical (86% pure)	EC	42.00	24.07	10.78
	FI	42.08	20.37	7.12
	CC	32.75	19.03	7.52
Analytical (99+% pure)	EC	0.03	0.04	0.02
	FI	0.03	0.09	0.03
	CC	0.02	0.06	0.03

tions 1 and 2. Solution 1 contained di-, tri-, and tetraCDD. Solution 2 contained hexa- (four isomers), hepta- (two isomers), and octaCDD. All quantitation was based on peak heights.

A precipitate from fraction III of the technical product was analyzed by infrared spectrophotometry using a Perkin-Elmer 521 and a multiple internal reflection apparatus (Wilks Scientific Corporation, 1965).

Mass spectral analysis of fraction III of the technical product was accomplished using an LKB 9000 equipped with a mass marker (±0.3 mass unit) and interfaced to a 10 ft × ¼ in. o.d. coiled pyrex glass column packed with 3% SE-30 on 60/80 Chromosorb G, DMCS treated, AW. The column was maintained at 230° with a helium flow of 70 cm³/min at 12 psi. Under these conditions the 2, 3, 7, 8-tetraCDD had a retention time of 9.5 min.

RESULTS AND DISCUSSION

The data obtained by gc analysis of fractions III and IV from the technical and pure PCP extracts are summarized in Table I. Although fractions III and IV and the precipitate were analyzed separately, the data are combined in Table I. Values are reported for the three modes of detection used. The highest CDD level was found in the technical grade PCP, where the order of decreasing concentrations of the CDD's present was hexa-, hepta-, and octaCDD. In the pure sample of PCP, heptaCDD was higher in concentration than hexa- or octaCDD. The technical product was more contaminated than the analytical one by factors of 1400, 600, and 539 for the hexa-, hepta-, and octaCDD, respectively.

An infrared spectrum of the precipitate from the technical product fraction III is presented in Figure 1. The spectrum of this residue is superimposable on the spectrum of an octaCDD standard (Woolson *et al.*, 1972). Gas chromatographic analysis showed this residue to be predominantly octaCDD.

Mass spectral analysis confirmed the presence of the reported CDD's. TetraCDD, the most toxic of the CDD's, was not found in the PCP's analyzed. The minimum detectable limit was 0.4 ppm.

Mass spectral analysis also revealed the presence of several chlorodibenzofurans (CDF's) and chlorodiphenyl ethers (CDE's). Hexa-, hepta-, and octaCDF and hexa-, hepta-, and octaCDE were found as original constituents of PCP. The mass spectra of the hexa- and heptaCDE contained additional ions of 304 and 338, which correspond to the masses of tetra- and pentaCDF, respectively. Multiple scans of these two CDE's indicated that tetra- and pentaCDF were formed subsequent to the loss of two chlorines from the parent CDE's. It is conceivable that *o,o'*-substituted CDE's could rearrange to CDF's upon fragmentation.

Gas chromatography–mass spectrometry indicated that the CDD's were not completely resolved from the CDF's and CDE's. Consequently, the calculations of CDD levels, which were based on CDD standards alone, may be slightly high. HexaCDD showed more interference from CDF and CDE than did hepta- and octaCDD.

ACKNOWLEDGMENT

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No. 414

Characterization of Alkylation Products of Diethyl Phosphorothioate

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The methylation of *O,O*-diethyl phosphorothioate (DETP) produced a minor component identified as the *O,O*-diethyl-*O*-methyl phosphorothionate (DEMMTP) (15%) and a major product identified as the *O,O*-diethyl-*S*-methyl phosphorothiolate (DEMMPTh) (85%). The ethylation also produced two compounds which were identified as *O,O,O*-triethyl phosphorothionate (TETP) (37.5%) and *O,O,S*-triethyl phosphorothiolate (TEPTh) (62.5%). Alkylation of *O,O*-dimethyl phosphorothioate has never produced a detectable amount of trialkylated thiolate. The compounds were identified by comparing the gas chromatograms obtained with an instrument equipped with a flame photometric detector and the mass spectra with those of the purified standards TETP, TEPTh, DEMMTP, and DEMMPTh. In a previous publication concerning the analysis of hydrolytic and metabolic products of organophosphorus pesticides, the compounds labeled as ethylated or methylated DETP should be labeled ethylated or methylated *O,O*-diethyl phosphorothiolate (DEPTh).

The gas chromatographic analysis (glc) of the methyl esters of *O,O*-dimethyl or *O,O*-diethyl phosphate, phosphorothioate, and phosphorodithioate has been discussed in the recent publications mentioned below. The reported order of elution of the methylated compounds by glc using a 6-ft column packed with 2% Ucon polar on 80- to 100-mesh Gas Chrom Q was: *O,O*-dimethyl phosphate (DMP); *O,O*-diethyl phosphate (DEP); *O,O*-dimethyl phosphorodithioate (DMDTP); *O,O*-dimethyl phosphorothioate (DMTP); *O,O*-diethyl phosphorodithioate (DEDTP); and *O,O*-diethyl phosphorothioate (DETP) (St. John and Lisk, 1968).

Askew *et al.* (1969) described another gas chromatographic technique based on the detection of methylated hydrolysis products of organophosphorus compounds. In this work, the dialkyl phosphate standards were prepared by hydrolysis of the parent organophosphorus pesticides which, upon methylation with diazomethane, produced the various trialkyl phosphates. The methylated dialkyl phosphates were separated on silica gel thin-layer plates, eluted with chloroform, and examined by glc to show that one component was present. The structure of the phosphate esters was confirmed by infrared spectroscopy. The reported glc elution pattern of the methylated compounds employing a 150-cm column packed with 4% Versamid 900 was in the following order: DMP, DEP, DMTP, and DETP.

A recent publication describes the use of a 12-ft column packed with 20% Versamid 900 for the simultaneous determination of six major metabolites of organophosphorus and thiophosphorus insecticides (Shafik and Enos, 1969). The suggested order of elution of the methylated or ethylated compounds was: DMTP, DMP, DEP, DMDTP, DEDTP, and DETP.

Recent mass spectral data obtained in this laboratory (Biros *et al.*, 1970) on purified standard materials suggested that the glc peak assignments for the methyl and ethyl derivatives of DETP reported previously were erroneous, and that these compounds were, in fact, *S*-alkylated derivatives of *O,O*-diethyl phosphorothiolate (DEPTh). The purpose of this investigation was to verify the structure of the alkylated diethyl phosphorothioates obtained from the reactions of the free acids with diazomethane and diazoethane, and to determine the relative proportion of *O*-alkylation and *S*-alkylation which may occur during the alkylation procedure.

EXPERIMENTAL

Standards

O,O-dimethyl phosphorothioate potassium salt	(DMTPK)
O,O,O-trimethyl phosphorothionate	(TMTP)
O,O-dimethyl phosphorodithioate	(DMDTPK)
O,O,S-trimethyl phosphorodithioate	(TMDTP)
O,O-diethyl phosphorothioate potassium salt	(DETPK)
O,O,O-triethyl phosphorothionate	(TETP)
O,O-diethyl phosphorodithioate potassium salt	(DEDTPK)
O,O,S-triethyl phosphorodithioate	(TEDTP)
O,O,S-triethyl phosphorothioate	(TEPTh)
O,O-diethyl-*O*-methyl phosphorothionate	(DEMMTP)
O,O-diethyl-*S*-methyl phosphorothiolate	(DEMMPTh)

The potassium salts of the dialkyl phosphates were obtained from American Cyanamid Co., Princeton, N.J. The symmetrical trialkyl phosphates, phosphorothionates, phosphorothiolates, and phosphorodithioates were prepared and purified by Vero Beach Laboratories, Inc., Vero Beach, Fla.

The DEMMTP and DEMMPTh were obtained in purified form from the reaction products of the methylation of *O,O*-diethyl phosphorothioate by silica gel column chromatography. The experimental details of the column chromatography procedure, useful as a combination cleanup and separation, are discussed in a separate publication (Shafik *et al.*, 1970).

The reagents, extraction and alkylation procedures, and gas chromatographic analysis were the same as those used in the procedure for the determination of the metabolic and hydrolytic products of organophosphorus pesticide chemicals (Shafik and Enos, 1969). The apparatus was the same except that the aluminum gas chromatographic column ($^1/_4$-in. o.d. × 12 ft) was replaced with a glass column of the same size.

RESULTS AND DISCUSSION

The methylation reaction of DMTPK and DMDTPK with diazomethane resulted in the formation of two compounds which produced identical glc retention times, and mass spectra as the purified standards TMTP and TMDTP, respectively.

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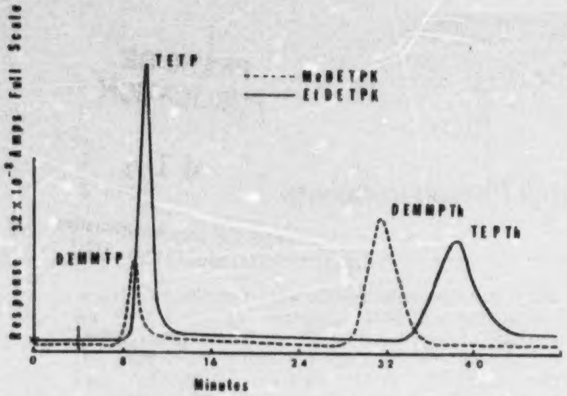

Figure 1. Chromatograms of methylated and ethylated products of 25 ng of DETPK, using the phosphorus filter, 526 mμ

No evidence of internal rearrangement was noted for these compounds. The ethylation of DEDTPK produced a trialkyl phosphate that had a mass spectrum and glc retention time identical to those of the purified standard TEDTP.

The alkylation of the available DETPK standard was examined after methylation and ethylation. Glc of the methylated standard showed the presence of two compounds; the early eluting derivative was a minor component and the later eluting derivative was a major component. The ethylated standard produced two major components (Figure 1). Glc of the two purified standards TETP and TEPTh, whose identities have been verified by mass spectrometry (Biros *et al.*, 1970), produced peaks which coincided with the two major components of the ethylated DETPK; the first was determined to be TETP and the second TEPTh.

The quantitation of the resulting two components after the ethylation of DETPK indicated that the reaction produced 37.5% TETP and 62.5% TEPTh.

Alkylation of DETPK with higher diazoalkanes, such as *n*-propyl, isobutyl, amyl, and isoamyl, produced one major component DETPR and a minor derivative DEPThR (R = *n*-propyl, isobutyl, amyl, or isoamyl). The amount of the minor component DEPThR decreased as the size of the R group increased. *S*-alkylation predominates on methylation of DETPK, but *O*-alkylation occurs preferentially with the higher diazoalkanes.

The methylation of DETPK produced two compounds in an 85 to 15 ratio. These components were separated by silica gel column chromatography and, by analogy with the mass spectra and relative glc retention times of the ethylated derivatives of DETPK, were shown to be DEMMPTh (85% isomer) and DEMMTP (15% isomer). The minor component DEMMTP cannot be quantitated under actual sample conditions as it will be masked by the large amount of inorganic phosphates which may be present in reagents and in urine samples. The inorganic phosphate is converted to TMP in the analytical procedure.

After methylation, the DETP is determined as DEMMPTh. Thus, in our previous publication (Shafik and Enos, 1969) the compound labeled DETP should be labeled DEPTh. At the present time it is impossible to determine the origin of *O*,*O*-diethyl-*S*-methyl phosphorothiolate by this method.

Quantitation of standards and unknown samples is based on the methylated DEP, DMDTP, DMDTP, DETP, and ethylated DMTP and DMP, using the phosphorus mode of the flame photometric detector. The chromatograms of the six methylated and ethylated alkyl phosphates are shown in Figure 2. The two derivatives DEMMTP and TMP that have identical retention times can be separated quantitatively by solvent partitioning. The details of the separation are presented, together with a cleanup procedure for the determination of low levels of alkyl phosphates in urine (Shafik *et al.*, 1970).

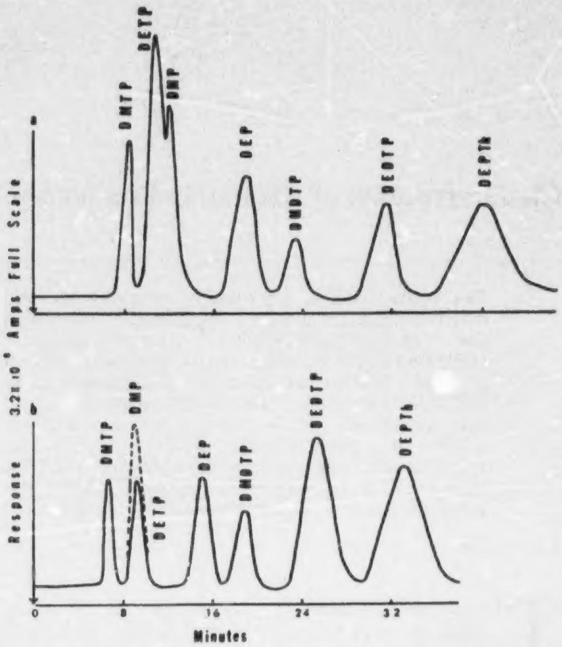

Figure 2. Chromatograms of trialkyl phosphate esters using the phosphorus filter, 526 mμ. (*a*) Ethylated: DMTP, 10 ng; DETP, 5 ng; DMP, 10 ng; DEP, 5 ng; DMDTP, 50 ng; DEDTP, 10 ng; DEPTh, 5 ng. (*b*) Methylated: DMTP, 10 ng; DETP, 10 ng: DMP, 10 ng; DEP, 5 ng; DMDTP, 50 ng; DEDTP, 5 ng; DEPTh, 5 ng

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Comparative Acceleration of Lindane Metabolism to Chlorophenols by Pretreatment of Rats with Lindane or with DDT and Lindane*

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Abstract—The stimulation of [^{14}C]lindane ([^{14}C]-γ-hexachlorocyclohexane) metabolism in rats by pretreatment with DDT or lindane has been previously demonstrated in this laboratory. However, it appeared that the mechanism by which lindane induced its own degradation differed from the mechanism by which DDT stimulated lindane metabolism. The present study attempted to clarify some of the differences in the metabolism of lindane resulting from pretreatment of rats with DDT and with lindane itself. Daily treatment of rats with either lindane alone or with DDT plus lindane permitted an evaluation of the comparative urinary excretion of the lindane-derived chlorophenols. Rats receiving DDT plus lindane excreted significantly more 2,4,5-trichlorophenol and 2,3,4,6- and 2,3,4,5-tetrachlorophenols by the second day of treatment than did rats receiving lindane alone. Results of the study suggest that DDT treatment stimulates the metabolism of lindane through a selective effect on certain metabolic pathways involved in the oxidative degradation of lindane.

INTRODUCTION

In 1966, 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane (DDT) was observed to antagonize the storage of 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-*exo*-1,4-*endo*-5,8-dimethanonaphthalene (dieldrin) in rats (Street & Blau, 1966) and other mammals (Street, Chadwick, Wang & Phillips, 1966). One year later, it was demonstrated that this response to DDT was the consequence of an increased degradation of dieldrin to polar metabolites (Street & Chadwick, 1967). Similarly, Koransky, Portig, Vohland & Klempau (1964) found that pretreatment of rats with phenobarbitone produced a marked acceleration in lindane (γ-hexachlorocyclohexane) elimination. Both groups observed that the increased metabolism was accompanied by an unspecified change in the types of metabolite excreted.

Barbiturates and miscellaneous drugs, including organochlorine insecticides, are believed to bring about a generalized non-specific induction of microsomal enzymes through similar mechanisms (Mullen, Juchau & Fouts, 1966). This laboratory is currently testing this hypothesis by characterizing the alterations that result in lindane metabolism when experimental animals are pretreated with various enzyme-inducing agents.

Earlier work in this laboratory suggested that rats pretreated with DDT metabolized a single oral dose of [^{14}C]lindane in a manner both quantitatively and qualitatively different from that observed in rats pretreated with lindane (Chadwick, Cranmer & Peoples, 1971), but the metabolites were not identified. In the same study, rats pretreated with DDT appeared to metabolize a dose of lindane in a manner similar to that of rats pretreated with DDT plus lindane. In the meantime, five previously unreported lindane metabolites have been isolated from rat urine and identified (Chadwick & Freal, 1972). Since the previous work indicated that pretreatment with DDT differed little from that with DDT and lindane in its effect on the metabolism of a single oral dose of [^{14}C]lindane, the rats in the study reported here were pretreated with DDT and lindane or with lindane so as to compare the acceleration of lindane metabolism to specific chlorophenols and other metabolites excreted in the urine.

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EXPERIMENTAL

Animals and diet. Eighteen weanling female Sprague–Dawley rats, randomly assigned to one of three groups, were housed in individual metabolism cages so that urine and faeces could be collected quantitatively. The feed used in this study consisted of a purified vitamin A test diet, obtained from Nutritional Biochemical Corporation. A vitamin supplement was administered in the drinking water.

Treatment. The three groups, each of six animals, were given their respective treatments by daily oral injection of peanut oil solutions, one group receiving 2 mg lindane daily, the second 2 mg each of both DDT and lindane, and the control group only the peanut oil. On day 7, all animals received 2 mg lindane containing 1·6 μc ^{14}C-labelled lindane in place of the previous treatment, after which treatment was discontinued.

Analysis of urinary metabolites. Throughout this experiment, 24-hr urine samples were collected and analysed for chlorophenols. Urine samples were individually acidified to pH 2·0 and extracted 3 times with equal volumes of benzene. The combined benzene extracts were then partitioned with a volume of 0·1 N-NaOH equivalent to 20% of the volume of the organic phase. The 0·1 N-NaOH was then acidified and extracted with an equal volume of benzene. This extract was analysed on a Tracor MT-220 gas chromatograph equipped with a Coulson electrolytic conductivity detector operated in the oxidative mode. The column used for the analysis consisted of a 6 ft × 0·25 in. glass U-tube packed with 5% DEGS on 80/100 mesh Gas Chrom Q, isothermally maintained at 180°C. The flow of nitrogen carrier gas was regulated at 90 cm^3/min.

Estimation of excreted radioactivity. On day 8 both urine and faeces samples were analysed for radioactivity. The radioactivity of the benzene extract of the urine, the 0·1 N-NaOH and the residual urine was determined separately. Dried samples of faeces were assayed for radioactivity by the oxygen-flask combustion method (Davidson & Oliverio, 1967; Oliverio, Denham & Davidson, 1962). Radioactivity of all samples was determined with a Nuclear-Chicago Mark I liquid scintillation counter.

Data evaluation. Designed comparison statistical methods were used to aid in the interpretation of the data (Snedecor, 1956). Unless otherwise indicated all comparisons in this study were considered significantly different at $P < 0·05$.

RESULTS

The radioactivity determined on day 8 in the urine extract obtained with 0·1 N-NaOH (chlorophenols), in the benzene extract (neutral metabolites), in the residual urine and in faeces from the control rats and those pretreated with lindane or DDT plus lindane is summarized in Fig. 1. The control, lindane and DDT plus lindane groups excreted, respectively, about 38, 58 and 91% of the administered radioactivity. The treated animals excreted significantly more radioactivity than the controls in every fraction, including the faeces. Similarly the rats treated with DDT plus lindane excreted significantly more radioactivity than the lindane-treated animals in every fraction except the residual urine. As in previous work (Chadwick *et al.* 1971), the proportion of radioactivity representing the free chlorophenols was greater in the urine from the rats pretreated with DDT plus lindane than in that from the controls or lindane-pretreated animals. The reason for the difference becomes apparent when one examines the alteration in the excretion patterns of the chlorophenols due to treatment.

The excretion patterns of 2,3,5-, 2,4,5- and 2,4,6-trichlorophenol from the animals treated with lindane and DDT plus lindane are presented in Fig. 2. Although higher levels of all three trichlorophenols were consistently excreted by the animals pretreated with DDT plus lindane, only the excretion of 2,4,5-trichlorophenol was significantly greater than that in the lindane-pretreated rats and then only at $P < 0{\cdot}10$. The excretion values for the controls are given on day 8, 24 hr after lindane was administered to all the animals. The treated animals excreted significantly more of all three trichlorophenols than did the controls. Accelerated lindane metabolism rather than an accumulation of chlorophenols or chlorophenol precursors in the tissues was undoubtedly responsible for the increased excretion.

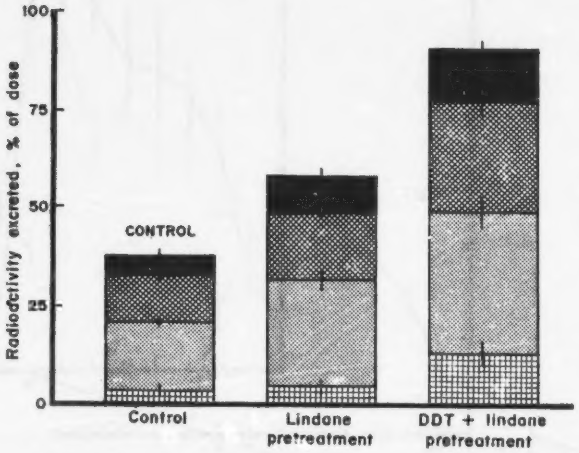

FIG. 1. Effect of no previous treatment (a) or of pretreatment with lindane (b) or with DDT plus lindane (c) on the excretion of radioactivity by rats after oral administration of [^{14}C]lindane. The indicated percentages of administered radioactivity appearing in the benzene extract after partitioning with 0·1 N-NaOH (neutral metabolites; ■), in the 0·1 N-NaOH extract (chlorophenols; ▩), in the residual urine (▦) and in the faeces (▦) for each treatment are mean values for six animals.

In previous work (Chadwick *et al.* 1971) a group of rats was pretreated with DDT for 2 wk and then given a single oral dose of 2 mg lindane (containing 2·0 μc ^{14}C-labelled lindane). It was found that besides excreting significantly more and storing significantly less radioactivity than the controls, the DDT-pretreated rats excreted significantly larger quantities of all of the subsequently identified chlorophenols. Since these animals received no lindane until the final day of the experiment, the increased chlorophenol excretion must have resulted from increased lindane metabolism. In the current study, treatment was discontinued on day 8, and within 24 hr the rats pretreated with DDT plus lindane excreted significantly smaller amounts of all three trichlorophenols than did all the other animals in the study.

As illustrated in Fig. 3, the effect of the pretreatments on the conversion of lindane to tetrachlorophenols was much more marked than was the case with conversion to trichlorophenols. By day 2, the rats treated with DDT plus lindane were excreting significantly more 2,3,4,5- and 2,3,4,6-tetrachlorophenol than were the lindane-treated animals. On day 8, the treated animals excreted significantly more of both tetrachlorophenols than did the controls. Moreover, those pretreated with DDT plus lindane excreted significantly more of both tetrachlorophenols than did the lindane-treated rats.

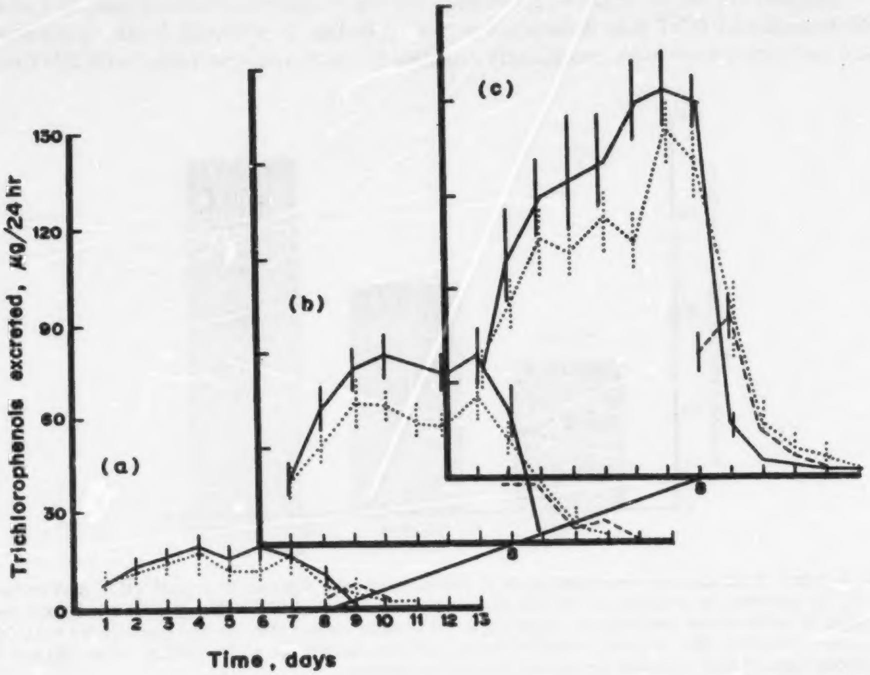

FIG. 2. Effect of pretreatment with lindane (· · · ·) or DDT plus lindane (———) on the daily urinary excretion of the (a) 2,3,5-, (b) 2,4,5- and (c) 2,4,6-trichlorophenol metabolites of lindane by rats. Each point represents the mean excretion value for six animals. On day 7, all animals, including those of the control group (– – – –), received lindane and treatment was then discontinued.

The excretion of the tetrachlorophenols on day 8 is shown in Fig. 4. In this case, the controls excreted less than 25% as much 2,3,4,5- as 2,3,4,6-tetrachlorophenol, compared with about 45% and nearly 80%, respectively, for the animals pretreated with lindane and with DDT plus lindane. The stimulation of lindane metabolism to 2,3,4,6-tetrachlorophenol by the DDT plus lindane pretreatment was a substantial 1·3 times that produced by

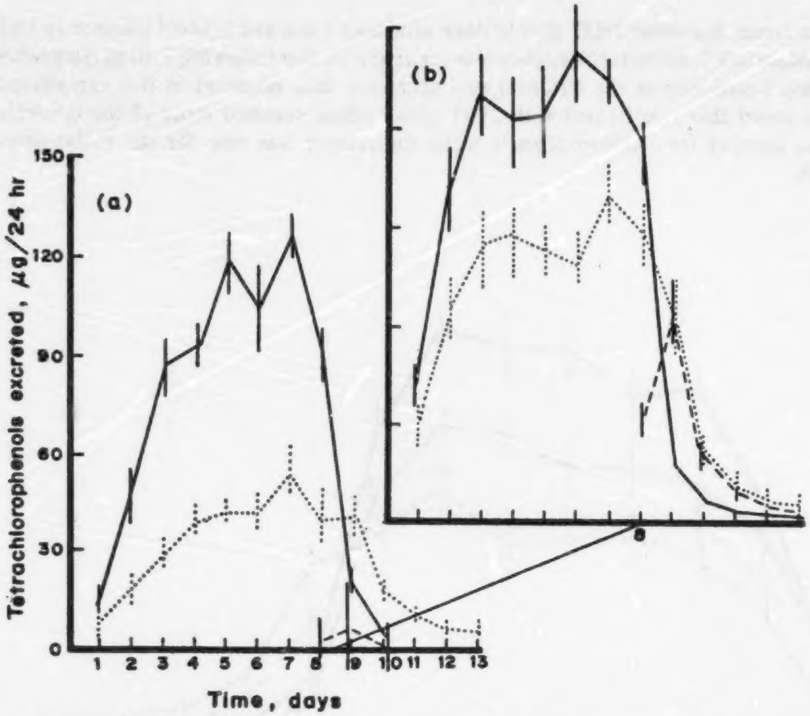

Fig. 3. Effect of pretreatment with lindane (····) or DDT plus lindane (———) on the daily urinary excretion of the (a) 2,3,4,5- and (b) 2,3,4,6-tetrachlorophenol metabolites of lindane by rats. Each point represents the mean excretion value for six animals. On day 7, all animals, including those of the control group (– – – –), received lindane and treatment was then discontinued.

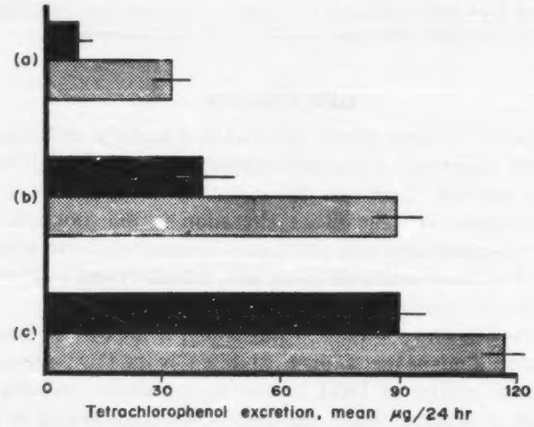

Fig. 4. Comparison of the total urinary excretion of lindane-derived tetrachlorophenols (2,3,4,5- isomer, ■; 2,3,4,6- isomer, ▥) on day 8 by rats given lindane on day 7 following no treatment (a) or pretreatment with lindane (b) or DDT plus lindane (c). Each bar represents the mean excretion for six animals.

lindane alone. However, DDT plus lindane produced a marked 2·3-fold increase in lindane metabolism to 2,3,4,5-tetrachlorophenol compared with that following lindane pretreatment.

Figure 5 summarizes the chlorophenol excretion data obtained in this experiment. It will be noted that rats treated with DDT plus lindane excreted more of the tetrachlorophenols than of the trichlorophenols while the reverse was true for the lindane-treated animals.

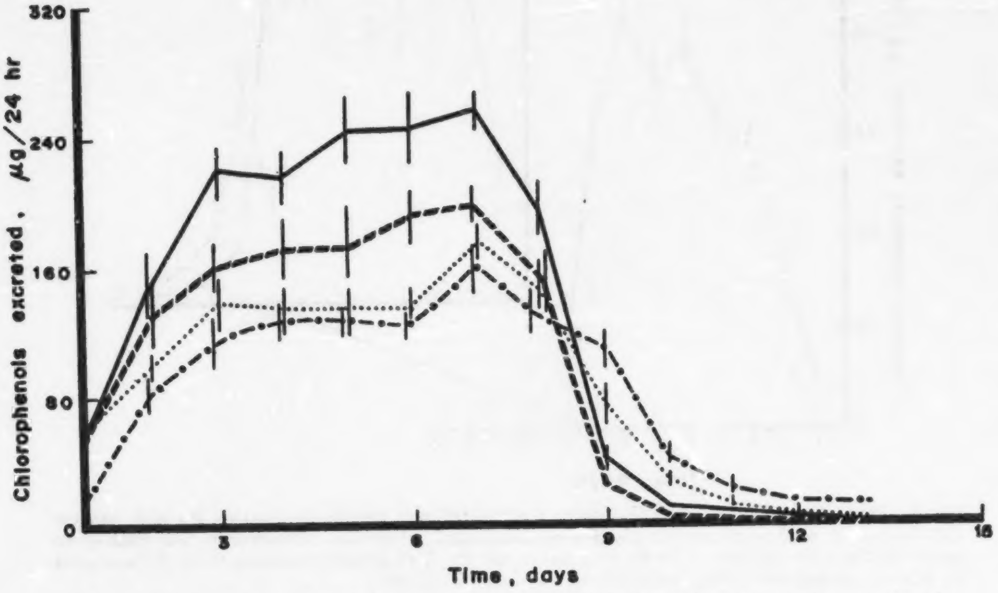

Fig. 5. Effect of pretreatment with lindane (–·– and · · · ·) or DDT plus lindane (– – – and ———) on the daily urinary excretion of total trichlorophenols (· · · · and – – – –) and total tetrachlorophenols (–·– and ———) by rats. Each point represents the mean excretion value for six animals.

DISCUSSION

The results of this study indicate that in addition to a uniform difference in total lindane metabolism, which one might expect from pretreatment of rats with similarly acting enzyme inducers of different potency, there are also quantitative differences in the excretion of certain specific metabolites. The significant alteration in the proportion of tetrachlorophenols excreted by rats pretreated with DDT plus lindane compared with that excreted by the lindane-pretreated animals is difficult to explain, if lindane and DDT stimulate metabolism by similar non-specific mechanisms.

From these observations and the data in this study, it appears reasonable to speculate that the greatly enhanced lindane metabolism exhibited by the DDT-pretreated rats resulted in part from the selective effect of DDT on certain metabolic pathways involved in the oxidative degradation of lindane. Such selectivity was demonstrated in this study by the marked stimulation of the enzymes involved in the metabolism of lindane to the tetrachlorophenols, particularly to 2,3,4,5-tetrachlorophenol.

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Accélération comparative de la métabolisation du lindane en chlorophénols chez le rat par un traitement préalable au lindane et au DDT et lindane

Résumé—Le laboratoire dont émane cette communication a démontré précédemment qu'un traitement préalable au DDT ou au lindane stimulait, chez le rat, le métabolisme du [^{14}C]-lindane ([^{14}C]-γ-hexachlorocyclohexane). On a toutefois constaté que le mécanisme par lequel le lindane déclenchait sa propre dégradation diffère du mécanisme par lequel le DDT stimule la métabolisation du lindane. On a donc tenté, par les expériences décrites ici, d'élucider quelques-unes des différences en métabolisme du lindane observées quand on soumet les rats à un traitement préalable, soit au DDT, soit au lindane même. L'administration quotidienne de lindane ou de lindane + DDT a permis de comparer et d'évaluer les excrétions urinaires des chlorophénols dérivés du lindane. Les rats qui recevaient du DDT et du lindane ont excrété le deuxième jour de traitement significativement plus de 2,4,5-trichlorophénol et de 2,3,4,6- et 2,3,4,5-tétrachlorophénols que ceux qui recevaient uniquement du lindane. Les résultats de ces expériences suggèrent que le traitement au DDT stimule le métabolisme du lindane *via* un effet sélectif sur certaines voies métaboliques impliquées dans la dégradation oxydative du lindane.

Vergleich der Beschleunigung des Lindanstoffwechsels zu Chlorphenolen durch Vorbehandlung von Ratten mit Lindan oder mit DDT und Lindan

Zusammenfassung—Die Anregung des Stoffwechsels von [^{14}C]Lindan ([^{14}C]-γ-Hexachlorcyclohexan) bei Ratten durch Vorbehandlung mit DDT oder Lindan wurde schon früher in diesem Laboratorium demonstriert. Es scheint jedoch, dass der Mechanismus, mit dem Lindan seine eigene Zerlegung einleitet, sich von dem Mechanismus unterscheidet, mit dem DDT den Lindanstoffwechsel anregt. Die vorliegende Untersuchung versuchte einige der Unterschiede zwischen dem Lindanstoffwechsel zu klären, der sich nach der Vorbehandlung von Ratten mit DDT und mit Lindan selbst ergibt. Tägliche Behandlung von Ratten entweder mit Lindan allein oder mit DDT plus Lindan erlaubte eine vergleichende Beurteilung der Ausscheidung der aus dem Lindan entstandenen Chlorophenole im Urin. Ratten, die DDT plus Lindan erhalten hatten, schieden wesentlich mehr 2,4,5-Trichlorphenol und 2,3,4,6- sowie 2,3,4,5-Tetrachlorphenol am zweiten Tag der Behandlung aus als Ratten, die nur Lindan erhalten hatten. Die Ergebnisse des Versuchs legen die Annahme nahe, dass die DDT-Behandlung den Lindanstoffwechsel durch selektiven Einfluss auf bestimmte Stoffwechselwege anregt, die beim oxydativen Abbau von Lindan in Anspruch genommen werden.

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DDT in the Diet
Of the Rat

Its Effect on DDT Storage, Liver Function And Cell Morphology

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Introduction

That the repeated ingestion of DDT at or near the toxic level should be the cause of histological changes in rats has never been a source of astonishment, and there is general agreement regarding the character of the changes and the fact that they occur predominantly in the liver (*1–6*). However, considerable interest was aroused in 1950 when, following a brief announcement by Kunze and associates (*7*), Laug and co-workers (*8*) reported that changes could be detected in the liver of rats maintained for 6 months on a diet containing 5 parts per million (p.p.m.) of DDT. Interest arose from the fact that this concentration of DDT was one which might occur—though certainly not frequently nor as an average value—in human diet. Interest also arose from the suggestion by Laug and his colleagues that the potential hazards of DDT had been underestimated.

In the following year, Cameron and Cheng (*9*) reported totally different results. As in earlier studies by Cameron and Burgess (*10*) of rats[1] fed large doses of DDT, these authors were unable to demonstrate any pathology in rats sacrificed after being dosed for more than a year at levels corresponding to food concentrations up to approximately 350 p.p.m.[2] They suggested that the changes attributed by others to the ingestion of small dosages of DDT were, in fact, fixation artifacts.

Although the work of Kunze, Laug, and their colleagues and the work of Cameron and Cheng are the only direct attacks on the problem, it should be mentioned that Deichmann and colleagues (*12*) and Treon and Cleveland (*13*) reported liver changes induced by relatively low dosages of DDT, while Haag and associates (*14*) and Greenwood and associates (*15*) failed to find such changes.

The effect of small, repeated intake of DDT has been reinvestigated because there is general agreement on the importance of the problem. Although their findings were negative, Cameron and Cheng (*9*) stated the matter as follows: "At the present time nothing is known about the action of DDT beyond the fact that it interferes in some way with the normal function of the nervous system. . . . Special importance must still be attached to histological evidence of tissue damage or change occurring in animals exposed to DDT."

The present study has been designed more nearly in accord with the methods of Laug and his colleagues (*8*) than with the methods of Cameron and Cheng (*9*). The possibility has not been excluded that the opposite results of the two groups were determined by the differences in their techniques. Laug and his associates incorporated DDT directly into chow which was taken ad libitum by the rats and served as their only source of food. Cameron and Cheng administered a peanut oil solution of DDT to the tongue of rats; the rats were then allowed free access to uncontaminated food. The method used by Laug and associates is certainly more nearly comparable to the kind of exposure which human beings might conceivably get, and it was partly for this reason that the method of Laug was chosen for further study.

The present investigation involves not only the question of the occurrence of morphological changes induced in the liver by DDT but also the questions of alteration of hepatic function and the storage of DDT-derived compounds.

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[1] Cameron and Burgess found extensive liver damage in other species given high oral dosages.

[2] The possibility that the estimates for corresponding food levels were too low has been considered (*11*).

Methods

The design of the four experiments covered in this report is summarized in table 1. The basic experiment, No. 1, involved the feeding of numerous dietary levels of DDT and the sacrifice of rats after several intervals of feeding and of recovery. In experiment 2, an effort was made to study control animals subjected to a minimum of contamination with DDT. Experiment 3 was concerned with the degree of variation in the histopathological changes in the liver and with the speed of development of liver changes following very large doses of DDT. Experiment 4 investigated the possibility that the observed variations in histopathology might be associated with differences in the composition of different samples of DDT.

The animals used in all experiments were Sherman strain white rats taken from a colony that has been carefully selected to resist *Salmonella* infection. The animals were individually caged and had food and water available at all times. Rats were started on experiments when they weighed about 100 gm. each. All animals were weighed once a week, except that those in experiment 1 were weighed and their food intake was recorded daily for the first month of feeding.

DDT was weighed as a powder and mixed with ground Purina[3] laboratory chow, using an electric mixer. The Purina chow served as the only food of the rats except those in group 1 of experiment 2; rats of this single group were fed Purina chow from weaning until the beginning of the experiment, after which they received a similar chow of English manufacture. The English chow, designated diet No. 41, was obtained through the generosity of Dr. F. J. Dyer of the Medical Research Council Laboratories, Holly Hill, Hampstead, London, N. W3. The food, which has been described by Lane-Petter and Dyer (*16*), was not guaranteed to be free of DDT, but several chemical analyses found it to contain less than 0.05 p.p.m., the limit of sensitivity of the method. All the animals in experiment 2, including those fed English chow, were strictly isolated from the regular experimental animals and from persons whose duties brought them in contact with concentrations of DDT greater than 5 p.p.m.

Most of the tests were made with a first sample technical DDT assaying 86.6 percent p,p'-isomer and having a setting point of 90.4° C. Other tests were done with a very similar second sample, which analyzed 87.8 percent p,p'-isomer and had a setting point of 89.2° C. A few tests were done with p,p'-DDT which had been recrystallized three times from ethyl alcohol; it analyzed 97.6 percent[4] p,p'-isomer and had a melting point of 107° C. During the years of experimentation, occasional spot checks were made on the degree of contamination by DDT of the basic diet. The values ranged up to 0.5 p.p.m. Samples of food to which DDT had been intentionally added were analyzed also and showed satisfactory agreement with the intended concentrations.

All chemical analyses, whether of food or tissue, were made by the Schechter-Haller method. For tissue, the modification described by Mattson and co-workers (*17*) was used.

Liver function was studied by the bromsulphthalein (BSP) method. A 0.1-percent solution was injected intraperitoneally at the rate of 0.025 ml./gm., and 30 minutes later blood was removed from the heart following light ether anesthesia. Color was developed in the plasma by 10-percent NaOH and was measured with a Lumetron model 402 EF photoelectric colorimeter at a wavelength of 575 mμ.

Samples for histological study were taken as rapidly as possible after the blood had been drawn, and then samples for chemical analysis were obtained. Animals in which the liver function was not studied were also lightly anaesthetized at the time of sacrifice. The abdomen was quickly opened and the aorta divided. It is believed that exsanguination accomplished in this way minimizes variation in recorded organ weights and promotes rapid

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[3] Product of the Ralston-Purina Co., St. Louis, Mo.

NOTE: Trade names are for purposes of identification only and do not constitute endorsement by the Public Health Service.

[4] Failure of the sample to analyze 100 percent is thought to reflect the limitations of the chemical method more than impurity of the sample.

Table 1. Outline of experiments

Group No.	Concentration of DDT in diet (p.p.m.)	Number of animals								Serial sacrifice at—
		Total		Died		Studied for—				
		Male	Female	Male	Female	Liver function	Organ weight	Storage	Pathology	
				Experiment 1–A						
1	[1] <0.6	4	4	0	0	6	8	8	8	0, 2, 4, and 6 months.
2	[2] 5	4	4	0	0	6	8	8	8	
3	[2] 15	4	4	0	0	6	8	8	8	
4	[2] 50	4	4	0	0	6	8	8	8	
5	[2] 200	4	4	0	0	6	8	8	8	
6	[2] 400	5	5	1	1	5	8	8	8	
7	[2] 400	1	1	0	0	0	2	2	2	
				Experiment 1–B						
1	[1] <0.6	7	7	1	0	0	4	13	13	0.5, 1, 2.5, 5, 8, and 14 months after a feeding period of 6 months. Group 7 was maintained on 15 p.p.m. of DDT during the recovery period.
2	[2] 5	7	7	0	0	0	4	14	14	
3	[2] 15	7	7	0	0	0	4	14	14	
4	[2] 50	7	7	2	1	0	4	11	11	
5	[2] 200	7	7	1	0	0	4	13	13	
6	[2] 400	6	6	0	1	0	4	11	11	
7	[2] 400	7	7	1	1	0	4	12	12	
				Experiment 2						
1	[3] ≤ 0.05	9	0	0	-----	0	0	9	9	0.5, 1, 2, 2.5, and 3 months.
2	[1] <0.6	5	5	0	0	0	0	10	10	0.5, 1, 2, 4, and 6 months.
3	[4] 5	5	5	0	0	0	0	10	10	0.5, 1, 2, 4, and 6 months.
				Experiment 3						
1	[4] 50	10	0	1	-----	0	0	0	9	Biopsy at 2 months, sacrificed at 6 months.
2	[4] 200	19	0	4	-----	0	0	0	19	Biopsy at 6 and 8 months, sacrificed thereafter.
3	[4] 400	10	0	1	-----	0	0	0	5	2, 4, and 6 months.
4	[4] 1,000	10	0	2	-----	0	0	0	8	1, 2, 3, and 6 weeks.
5	[4] 1,000+ 5,000	6	0	2	-----	0	0	0	4	After 3 weeks' exposure to 1,000 p.p.m. and up to 5 days exposure to 5,000 p.p.m.
				Experiment 4						
1	[2] 200	10	0	1	-----	0	8	8	9	2 males sacrificed at 2 months; all others at 4 months.
2	[4] 200	10	10	0	1	0	17	17	10	
3	[5] 200	10	10	0	0	0	18	18	10	
	Total..	178	104	17	5	35	121	210	241	

[1] Basic ground Purina chow diet.
[2] First sample technical DDT.
[3] Basic English chow diet.
[4] Second sample technical DDT.
[5] Pure *p,p'*-isomer.

fixation of highly vascular tissues. In early experiments, tissue was removed for microscopic study from the liver, spleen, and kidney; in later experiments, the liver alone was sampled, for at moderate or low dosage levels it was found that changes could be detected in that organ only. Fat was removed from the retroperitoneal space and from around the internal genitalia for chemical analysis of DDT and DDE (1,1-dichloroethane-2,2-bis(p-chlorophenyl) ethylene) content.

In experiment 3, liver biopsies were carried out in order to follow the progress of histological changes in individual animals. Studies of liver function or liver weight were not made on rats which were biopsied. Liver samples consisting of a small lobe or a liberal segment of a large lobe (5 percent or less of the liver) were taken through an abdominal incision. Intraperitoneal sodium pentobarbital in propylene glycol was used for anesthesia. Hemostasis was frequently greatly aided by the use of Gelfoam.[5] Although wound infection was encountered occasionally, the animals usually withstood the surgical procedure well.

In view of the criticism made by Cameron and Cheng (*9*) regarding the pathological techniques used in previous studies, particular attention was paid to this matter in the present investigation. Because of the possibility of post-mortem changes, no attempt was made to examine carefully animals which died spontaneously. The reported histological results are based exclusively on freshly killed animals or upon biopsy material.

Because of the low dosage levels chosen for most of the tests, little spontaneous mortality, or even nonfatal illness, was encountered. However, the possibility of liver necrosis (as observed by Cameron and Cheng) in the few animals which died spontaneously was not excluded. The higher dosage levels used in experiment 4 constitute an exception, for in this experiment massive doses were studied in an effort to produce the most severe tissue damage possible.

In the course of the experiments, a rather comprehensive survey of various fixatives and stains was undertaken in order to develop the simplest and most reliable methods for detecting and grading lesions. Suffice it to say concerning fixatives that various formalin and formalin-salt, alcohol, mercuric salt, chromate, picric acid, and osmium mixtures were used. A similar wide spectrum of stains was tried, including oversight, connective tissue, cytoplasmic granule, nuclear, fat and lipoid, and various cell product stains. However, the important point was to select the simplest, most reliable, and most reproducible methods. The routine finally adopted for minimal study was as follows:

After rapid dissection, the organ was placed in isotonic saline solution. If weighing was necessary, this was speedily done without squeezing the specimen or allowing it to dry. The initial tissue slices were cut as thin as possible without crushing by a razor edge, and were immediately plunged into a liberal quantity of fixative. The fixatives used routinely were acid-Zenker (Helly), Zenker-formol (Spuler or Maximow), and 10-percent formalin buffered to pH 7.0 by acid sodium phosphate and disodium phosphate.

After several hours of fixation, the increased tissue firmness permitted a second subdivision without the risk of crushing, and the further subdivision insured a maximum degree of rapid and uniform fixative penetration. Zenkerized material was fixed for a total of 5 to 7 hours, whereas the formalin was used 3 to 5 days. The Zenker-fixed tissue was washed overnight, stored in iodinated 80-percent ethyl alcohol for 2 days, dehydrated with dioxane, and imbedded in a paraffin wax, m.p. 56–58° C.[6]

Formalin specimens were divided into two groups. One was mordanted 2 weeks in 3 percent potassium dichromate and then dehydrated in dioxane and imbedded in paraffin. The other was placed directly into 70-percent then into 90-percent, and finally imbedded in 100-percent polyethylene glycol,[7] at a temperature of 46° C. All sections, both in Carbowax and paraffin, were cut at 6 micra.

Slides of Zenker-formol tissue were stained with Lillie's azure-eosinate at a pH of 8.0 or 9.0. The acid-Zenker slides were stained with Bensley's aniline acid fuchsin-aniline blue-orange G method. These and other techniques used in this study can be found in Lillie's text (*18*) unless otherwise stated. The Bensley technique requires some amplification if reproducible results are to be expected because the method is most capricious unless carefully controlled.

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[5] Product of the Upjohn Co., Kalamazoo, Mich.

[6] Fisher Tissuemat, Fisher Scientific Co., Silver Spring, Md.

[7] Carbowax 1000, Carbide & Carbon Chemicals Co., New York, N.Y.

First, the fixation must be acid-Zenker for best results. The stain must be prepared from well-preserved aniline. If the aniline has been permitted to remain at room temperature and to accumulate oxidation products, which give the compound a brown color, some fatty structures fail to stain. Both the pure aniline and the saturated solution of aniline in distilled water should be kept in a refrigerator in contact with aluminum wire. Before using the water solution in compounding, it should be brought to room temperature with an excess of aniline present. It is essential not to use the final aniline-acid-fuchsin mixture more than 1 month. Results were somewhat better if the staining period in aniline blue and orange G was shortened to 30 minutes. As a final check, a control slide was stained routinely along with the experimental ones.

The paraffin sections, primarily fixed in formalin and then mordanted in 3-percent potassium dichromate, were stained by a method similar to that of Ciacco, but using Lillie's supersaturated Oil Red O dye in isopropanol instead of Sudan IV. The Carbowax sections were handled, using the Rinehart and Abul-Haj (*19*) general method of Carbowax embedding and fat staining, but substituting Oil Red O for Sudan IV. All preparations stained for fat were mounted in an equal mixture of saturated solutions of gelatin and of glucose.

Results

Morbidity and Mortality

Severe, often unremitting, tremors were seen in rats fed at 1,000 p.p.m. At levels of 200 and 400 p.p.m., both males and females were often nervous and hyper-reactive; however, severe tremors were usually of short duration and spontaneously remitted.

In experiment 1, involving 148 rats, only 2 animals, a male and a female, died during the 6-month course of treatment. Both were treated at a level of 400 p.p.m., but only the female showed neurological symptoms at the time of death. The male showed pneumonia. During the subsequent 14 months of recovery, 5 males and 3 females died, apparently of intercurrent infections; they showed no signs of DDT poisoning prior to death. However, all but a single control animal had received 50 p.p.m. or more, and 5 of the total of 10 deaths occurred at the level of 400 p.p.m. In the other experiments, mortality was occasionally caused by surgery or by infection as the animals became advanced in age. In view of the duration of exposure, mortality from DDT poisoning was appreciable in the 1,000-p.p.m. group only, but, because the number of animals was small and the rats were frequently sacrificed, the mortality figures were not informative. Mortality from DDT poisoning, intercurrent infection, and surgery is summarized without distinction in the appropriate column of table 1.

Weight Gain

Rats in experiment 1 were weighed daily for the first 35 days of exposure, a period in which they were growing rapidly. The ingestion of DDT in the diet at a rate of 400 p.p.m. or less produced no change in the growth rate of experimental rats as compared to rats on the control diet. The results from other experiments, involving levels up to 400 p.p.m., were consistent. All rats fed at 1,000 p.p.m. or more showed anorexia and striking weight loss.

Liver Function

Liver weights were recorded for rats in experiments 1 and 4. The results, shown in table 2, indicate a definite increase in the weight of the liver in both male and female rats fed DDT at the rate of 400 p.p.m. The liver weight, expressed as percentage of body weight, was 5.2 and 5.3, for male and female rats, respectively, after a feeding period of 2 months. These figures may be compared with 5.7 percent found by Sarett and Jandorf (*20*) for female rats fed 700 p.p.m. for 36 to 40 days. The control value in the present experiment was 3.8, as compared with 3.7 observed by Sarett and Jandorf.

In experiment 1, the change in liver weight in rats fed at 400 p.p.m. was most marked after 2 months of exposure. A partial return to normal in the face of continued exposure was suggested but not conclusively demonstrated. Evidence for a significant change in liver weight was lacking in rats fed smaller concentrations.

BSP determinations, made after 2 and 4 months of exposure to varying levels of DDT up to 400 p.p.m., showed no significant change in dye retention at any dose. In terms of milligrams of BSP/100 ml. of plasma, the mean control value was 1.15, whereas that for the DDT-treated animals as a whole was 1.20. Treating each dosage level individually gave no significant difference from the initial value.

To illustrate the sensitivity of the method when dealing with potent hepatotoxic agents, the following figures are quoted for the identical technique used with carbon tetrachloride poisoning. A moderate dose of CCl_4, given subcutaneously at the rate of 6,450 mg./kg. of carbon tetrachloride in olive oil, produced less than a 10-percent mortality. The control BSP value for this series of animals was 1.00 mg./100 ml., whereas the mean value for treated animals was 3.85. Because of the uniformly negative results found in the DDT series, the BSP test was discontinued.

Table 2. Liver weight in relation to concentration of technical DDT in the diet and duration of feeding

(Experiments 1 and 4)

DDT in diet (p.p.m.)	Sex	Liver weight as percentage of body weight				
		Months on diet			Months recovery following 6 months of exposure	
		2	4	6	0.5	1
<0.6	Male	3.8	2.7	3.2	3.5	3.3
	Female	3.8	3.6	3.3	4.2	3.2
5	Male	3.6	3.1	2.9	3.0	3.2
	Female	3.8	3.6	2.9	4.0	3.5
15	Male	3.5	3.1	3.1	3.0	2.9
	Female	3.8	3.2	3.2	3.4	3.7
50	Male	4.0	3.2	3.6	3.1	3.6
	Female	4.4	3.4	3.6	3.3	3.7
200	Male	[1] 3.8	[2] 3.5	4.1	4.2	3.2
	Female	4.0	[3] 3.2	3.4	4.4	3.5
400	Male	5.2	4.2	3.4	3.9	3.7
	Female	5.3	4.2	4.3	4.1	4.0
400	Male	----------	----------	4.0	[4] 4.1	[4] 4.7
	Female	----------	----------	4.7	[4] 4.0	[4] 3.8

[1] Based on 6 animals; standard error, 0.59. [2] Based on 25 animals; standard error, 0.55. [3] Based on 20 animals; standard error, 0.77. [4] Continued to feed DDT at 15 p.p.m. during "recovery" period.

Table 3. Technical DDT in rat fat in relation to concentration of technical DDT in the diet and to duration of feeding and recovery

(Experiment 1)

DDT in diet (p.p.m.)	Sex	Technical DDT (p.p.m.)								
		Months on diet			Months recovery following 6 months of exposure					
		2	4	6	0.5	1	2.5	5	8	14
<0.6	Male	7	38	18	24	20	12	28	11	18
	Female	17	28	19	24	20	12	20	12	14
5	Male	56	58	70	67	42	44	25	18	20
	Female	61	111	102	70	84	56	41	26	31
15	Male	106	140	115	65	100	61	39	20	18
	Female	174	219	337	358	151	115	98	30	37
50	Male	134	326	234	203	101	113	72	17	-------
	Female	288	493	459	306	650	375	127	119	56
200	Male	802	534	540	590	309	244	86	34	48
	Female	1, 720	1, 163	4, 190	842	1, 160	455	634	226	45
400	Male	843	1, 352	896	496	618	283	158	39	70
	Female	2, 620	1, 162	3, 028	2, 850	730	1, 120	309	177	-------
400	Male	-------	-------	880	[1] 580	[1] 647	[1] 280	[1] 180	[1] 144	[1] 230
	Female	-------	-------	5, 130	[1] 2, 598	[1] 2, 980	[1] 687	[1] 593	[1] 335	[1] 284

[1] Continued to feed DDT at 15 p.p.m. during "recovery" period.

Tissue Storage of DDT and DDE

The storage data obtained in experiment 1 are shown in tables 3–6. The following conclusions regarding DDT are based on table 3. DDT is stored in the fat of both male and female rats. The amount of storage increases with the dosage, but as a general trend, a smaller proportion of the intake is stored at higher than at lower dosage levels.

Female rats regularly stored more DDT than comparable male rats. Furthermore, as shown in table 6, this tendency was accentuated at high dosage levels. During the feeding period, the female/male ratio was 1.5 and 3.0 for rats fed 5 and 400 p.p.m., respectively.

Regarding the rate of storage, 41 percent or more (average 61 percent) of the greatest observed storage was accomplished during the first 2 months of exposure. At all feeding levels, male rats reached or almost reached storage equilibrium in 4 months. The same was true for females fed 50 p.p.m. of DDT or less, but females fed 200 and 400 p.p.m. showed further accumulation of DDT in the fat after 4 months of exposure. It is not possible, on the basis of available data, to determine whether female rats fed 200 p.p.m. or more of DDT require more than 6 months of exposure in order to reach equilibrium of DDT storage.

When exposure to DDT was terminated, there was a continuous loss of DDT from the fat, irrespective of sex or dosage level. Control animals showed DDT storage in the fat ranging from 7 to 38 p.p.m. and averaging 18 p.p.m. Male and female rats previously fed at 5 p.p.m. and 15 p.p.m. lost DDT so that 8 months after the last exposure their storage values were not significantly different from the control values. The same was true of male rats fed at 50 p.p.m. or less. Female rats fed at 50 p.p.m., and all rats fed at 200 p.p.m. and 400 p.p.m. failed to return completely to a control value during the 14-month observation period. However, the rate of loss was most rapid at high storage levels, amounting to more than 90 percent in 14 months. Although the rats at high dosage levels did not return to control values, they lost more DDT, both in absolute terms and in terms of percentage of the maximum amount stored, than did rats at low dosage levels. In a similar way, females lost a higher percentage of DDT in a given period than males fed at the same rate because the females stored more of the compound.

Rats fed for 6 months on 400 p.p.m., and then placed on a diet containing 15 p.p.m. (group 7 of experiment 1–b) lost stored DDT, so that the concentration remaining in the fat after 8 months was about equal to the largest concentration observed in rats fed on 15 p.p.m.

The storage of DDE by animals in experiment 1 is shown in table 4. Male rats on the control diet, which contained a trace of DDT, stored an average of 2 p.p.m. of DDE during the first 6 months of the experiment. During the same period, the amount of DDE stored by male rats fed technical DDT at concentrations up to 400 p.p.m. was also very small. The figures fail to establish that there was an increase in DDE storage with increasing DDT dosage, although the possibility of a very small increase is not excluded. The female rats, on the contrary, showed a very definite increase in the storage of DDE correlated with increasing dosage of DDT.

Male rats on the control diet stored slightly more DDE during the latter 14 months of the experiment than during the first 6 months. There is evidence that male rats previously fed on 5 to 400 p.p.m. of DDT stored slightly more DDE after feeding was discontinued (average 11.9 ± 1.9 p.p.m.) than during the period of dietary exposure (average 4.2 ± 0.9 p.p.m.). The results are consistent with those of Smith and associates (*21*), who found that DDE is excreted more slowly than DDT. Because storage equilibrium of DDE was probably not achieved during the feeding period, and because DDE may be formed, in part, from stored DDT, it does not appear possible, from available storage data, to analyze further the rate of DDE loss.

The male rats originally fed at 400 p.p.m., and later changed to 15 p.p.m., showed little

Table 4. *o,p*-plus *p,p'*-DDE in rat fat in relation to concentration of technical DDT in the diet and to duration of feeding and recovery

(Experiment 1)

DDT in diet (p.p.m.)	Sex	DDE (p.p.m.)								
		Months on diet			Months recovery following 6 months of exposure					
		2	4	6	0. 5	1	2. 5	5	8	14
<0.6	Male	2	3	1	6	3	4	13	10	10
	Female	7	6	7	8	4	4	9	12	7
5	Male	5	3	8	4	3	12	7	6	10
	Female	10	8	19	11	19	43	15	11	27
15	Male	7	3	4	2	8	9	14	8	11
	Female	30	11	26	74	22	23	47	22	31
50	Male	9	0	0	15	5	9	12	8	------
	Female	11	38	20	52	140	85	23	77	30
200	Male	0	0	0	13	0	9	14	9	34
	Female	190	57	1, 180	68	370	206	232	115	69
400	Male	8	8	8	0	50	9	28	9	27
	Female	280	20	822	1, 130	180	500	155	117	------
400	Male	------	------	30	[1] 0	[1] 0	[1] 11	[1] 5	[1] 14	[1] 9
	Female	------	------	1, 630	[1] 400	[1] 1, 100	[1] 283	[1] 302	[1] 285	[1] 270

[1] Continued to feed DDT at 15 p.p.m. during "recovery" period.

Table 5. Percentage DDE of total insecticide-derived material found in rat fat in relation to concentration of technical DDT in the diet and duration of feeding and recovery

(Experiment 1)

DDT in diet (p.p.m.)	Sex	Percent DDE								
		Months of diet			Months recovery following 6 months of exposure					
		2	4	6	0. 5	1	2. 5	5	8	14
<0.6	Male	22	7	5	20	13	27	32	18	36
	Female	29	18	26	24	17	25	45	50	39
5	Male	8	6	10	6	7	21	21	25	35
	Female	14	7	16	13	18	44	27	30	47
15	Male	6	2	4	3	7	13	26	29	37
	Female	15	5	16	17	13	17	32	42	45
50	Male	6	0	0	7	5	7	14	32	------
	Female	4	7	4	15	18	18	15	39	35
200	Male	0	0	0	2	0	4	14	21	41
	Female	10	5	22	7	24	31	27	34	61
400	Male	1	1	1	0	8	3	15	19	28
	Female	10	2	21	28	20	31	33	40	------
400	Male	------	------	3	[1] 0	[1] 0	[1] 4	[1] 3	[1] 9	[1] 4
	Female	------	------	24	[1] 13	[1] 27	[1] 29	[1] 34	[1] 46	[1] 49
Mean [2]	Male	6	2	3	3	5	9	16	22	30
	Female	11	5	17	16	20	28	28	40	46
Ratio of means	$\frac{\text{Female}}{\text{Male}}$	1. 8	2. 5	5. 7	5. 3	4. 0	3. 1	1. 8	1. 8	1. 5

[1] Continued to feed DDT at level of 15 p. p. m. during "recovery" period. [2] Omitting control values.

difference from other groups in the storage of DDE, but comparable female rats maintained their DDE storage level more persistently than any other group studied.

Experiment 1

For animals in experiment 1, the percentage of DDT-related material found to be DDE is shown in table 5. As could be expected, the percentage of DDT-related material stored by the male rat as DDE varied inversely with the DDT dosage. For the female, the percentage of DDT-related material stored as DDE varied little with the DDT dosage. Both sexes showed a tendency for the proportion of stored DDE to increase as the recovery period progressed, reflecting a slower overall loss of DDE than of DDT. The female/male ratio for the proportion of DDE increased from 1.8 to 2.5 to 5.7 during the feeding period, and then gradually decreased to 1.5 during the recovery period. This reflects the ability of the female to store relatively large amounts of DDE following exposure to high dosage levels of DDT.

Table 6 shows a comparison of the female/male ratios for the storage of DDT-derived materials during the feeding and recovery periods in relation to dosage. The greater ability of the female rat to store DDT and its very much greater ability to store DDE are clearly demonstrated.

Experiment 2

Experiment 2 concerned an attempt to obtain rats as nearly free of DDT as possible. The data on DDT storage obtained in this experiment are presented in table 7. All of the animals in this experiment were kept in an uncontaminated, air-conditioned laboratory, completely separate from other animals being exposed to DDT.

Table 6. Comparison of female/male ratios for storage of DDT-derived materials during feeding and recovery periods in relation to dosage

(Experiment 1)

DDT in diet (p.p.m.)	Total DDT+DDE		Technical DDT		DDE	
	Feeding period	Recovery period	Feeding period	2.5 to 14 months recovery period	Feeding period	2.5 to 14 months recovery period
<0.6	1. 2	1. 1	1. 0	1. 4	3. 0	1. 1
5	1. 5	1. 8	1. 5	1. 5	2. 4	2. 7
15	2. 4	2. 2	2. 0	2. 0	4. 4	2. 9
50	1. 9	2. 9	1. 8	2. 5	7. 7	5. 4
200	4. 4	3. 5	3. 8	2. 7	21. 6	8. 1
400	3. 6	5. 1	3. 0	3. 9	50. 9	14. 3
400	3. 6	[1] 3. 5	3. 0	[1] 2. 3	50. 9	[1] 29. 2

[1] Continued to feed DDT at level of 15 p.p.m. during "recovery" period.

Table 7. Storage of DDT and DDE in fat of male rats in relation to concentration of technical DDT in the diet and to duration of feeding

DDT in diet (p.p.m.)	Sex	Months on diet					
		0.5	1	2	3	4	6
		Technical DDT (p.p.m)					
0.05	Male	5. 4	4. 2	[1] 3. 5	[2] 2. 6	--------	--------
<.5	Male	4. 2	3. 7	8. 8	--------	4. 4	6. 1
	Female	5. 0	5. 3	6. 5	--------	5. 8	11. 1
5.0	Male	22. 1	36. 8	59. 9	--------	88. [illegible]	85. 6
	Female	29. 8	39. 4	73. 8	--------	12[illegible]. 0	176. 7
		DDE (p.p.m.)					
0.05	Male	1. 1	1. 8	[1] 1. 8	[2] 1. 8	--------	--------
<.5	Male	. 7	1. 5	--------	--------	2. 0	2. 0
	Female	1. 5	1. 9	3. 2	--------	2. 9	1. 5
5.0	Male	. 7	1. 3	4. 8	--------	7. 1	1. 9
	Female	. 5	2. 4	5. 8	--------	6. 5	13. 4
		Percent DDT					
0.05	Male	17	30	34	41	--------	--------
<.5	Male	14	29	--------	--------	31	25
	Female	23	26	33	--------	33	12
5.0	Male	3	3	7	--------	7	2
	Female	2	6	7	--------	5	7

[1] Average of 5 animals. [2] Average of 2 animals.

Table 8. Storage of DDT and DDE in the fat and liver of male and female rats maintained on a concentration of 200 p.p.m. of DDT in their total diet for 4 months

(Experiment 4)

Value	Fat				Liver			
	Male		Female		Male		Female	
	DDT	DDE	DDT	DDE	DDT	DDE	DDT	DDE
	First sample technical							
Range	820–1, 262	el			16–34	1–3		
Mean	1, 042	el			21. 9	1. 6		
Standard error	54				2. 1	. 2		
	Second sample technical							
Range	773–1, 918	el	1, 254–4, 670	171–857	12–25	1–6	19–40	5–31
Mean	1, 156	el	2, 998	457	19. 8	2. 1	30. 7	13. 1
Standard error	123		383	85	1. 7	. 6	2. 1	2. 5
	Recrystallized							
Range	626–1, 539	el–318	1, 026–2, 541	213–851	8–18	2–7	14–20	4–11
Mean	949	[1] >134	1, 648	457	13. 8	4. 1	16. 4	6. 6
Standard error	111	[1] <39. 5	149	55	1. 7	. 7	. 7	. 6
	Total, all samples							
Range	626–1, 918	el–318	1, 026–4, 670	171–857	8–34	1–7	14–40	4–31
Mean	1, 042		2, 287	457	18. 0	2. 5	23. 2	9. 7
Standard error	57		249	48	1. 2	. 4	1. 9	1. 4

el—Experimental limit.

[1] In computing the mean and standard error, values below the experimental limit are taken as zero.

The rats in group 1 were fed chow of English manufacture. Because the supply of the special English food was limited, it could be used for only a small number of animals and for a short period. The animals fed on it undoubtedly entered the experiment with a small amount of stored DDT obtained under colony conditions. The storage values for these rats are consistent with the hypothesis that they stored no more DDT after entering the experiment but instead showed a progressive loss of stored material. However this may be, at the time of sacrifice, the rats fed on English chow were not free of DDT, and, in spite of the difference in trend, the amount which they contained was not surely distinguishable from the amount stored by rats fed on Purina chow during the same interval of the experiment. However, the amount of DDT stored by control rats fed on Purina chow in this experiment was significantly less than the amount stored by comparable rats in experiment 1. This observed difference appears to be a measure of the degree of contamination involved in feeding experiments employing large numbers of animals, some of which are maintained on high dosage levels.

The results for animals in experiment 2 fed at the rate of 5 p.p.m. showed satisfactory agreement with the values for comparable rats in the first experiment.

Experiment 4

Experiment 4 was designed to study possible differences in the action of technical and of pure DDT. Two samples of technical material were available for study. The results involving the storage of DDT-related compounds are shown in table 8.

Male rats fed pure crystalline DDT stored less DDT and more DDE in their livers than did either group of male rats fed technical DDT. The total amount of DDT-related materials stored in the liver was not significantly different for the three groups. The same trend—similar total storage involving less DDT and more DDE for rats fed recrystallized DDT—was evident in regard to the fat samples but in this instance the differences are not statistically significant. The females show the same trends for DDT but fail to show them for DDE.

Histopathological Studies

Studies were made on the spleen, kidney, and liver. No pertinent changes were observed in the spleen. Fatty infiltration of the proximal convoluted tubules was observed in rats fed diets containing 1,000 p.p.m. or more of DDT, but kidney changes were not observed in rats fed diets containing 400 p.p.m., or less.

The following changes were observed in the liver:

1. Fat.

1a. "Neutral fat" (studied with Lillie's supersaturated Oil Red O in isopropanol after fixation in 10-percent neutral formalin and imbedding in Carbowax 1,000).

1b. "Lipoid" (studied again with Lillie's Oil Red O technique using paraffin sections prepared from material fixed in 10-percent neutral formalin and then mordanted in 3-percent potassium dichromate).

2. Margination (studied with Lillie's buffered azure eosinate after Zenker-formol fixation).

3. Centrolobular cell hypertrophy (stained with azure eosinate after Zenker-formol fixation).

4. Inclusion body or liposphere formation (studied with Bensley's aniline acid fuchsin-orange G-aniline blue stain after acid Zenker fixation).

The histological appearance of liver tissue stained for neutral fat following the Carbowax method was similar to that after Ciacco's phospholipid technique in that both neutral fat and lipoid exist as discrete cytoplasmic globules. The affected liver cells were scattered in distribution when the changes were mild but tended to be midzonal when the changes were more severe. The "neutral" and "lipoid" types of fatty alteration refer primarily to the nonspecific accumulation of sudanophilic granules within the liver cell cytoplasm (fig. 1). Because of the predominantly fatty nature of lipospheres (which are described below and shown among others, in fig. 2) the fat content was likewise elevated when these bodies were frequent. However, it was the fat, differentiated only as granules, which was so frequent and so variable that its use as an index of tissue damage was impossible.

At higher dosage levels, margination was characterized not only by the peripheral position of the cytoplasmic granules but also by clumping of the granules and a diminution in their concentration; margination was characteristically associated with cell hypertrophy. The reality of both processes was confirmed by the freeze-drying technique.

Recorded cell hypertrophy was only partially due to the presence of lipospheres or fat. More typically, a mass of finely vacuolated or reticulated cytoplasm was visible which gave either a negative or a faintly diffuse reaction with Oil Red O. (See figs. 3–5 for the development of margination and hypertrophy following various dosages.)

The term "liposphere" (figs. 6 and 7) has been chosen to designate a characteristic, roughly spherical, complex, cytoplasmic inclusion with a fatty constitution found in rats exposed to DDT. When stained by routine histological methods, they may be distinguished only with difficulty from nonspecific hyaline inclusions described in the literature. Lipospheres consist of an external capsule of varying thickness, which takes a predominantly phospholipid stain, and of a central clear area which characteristically takes neutral fat stains.

When first identifiable, lipospheres occur as single bodies (down to 1μ in diameter) or as small clusters. As the dosage or the duration of exposure is increased, the structures increase in number, size, and thickness of the capsule.

Figure 1. Group of liver cells showing a nonspecific accumulation of neutral fat globules (× 2,800).

Male rat exposed to 15 p.p.m. DDT for 6 months. Oil Red O with hematoxylin counterstain after formalin fixation and Carbowax imbedding.

Figure 2. Liver stained to reveal lipoids. Fat intricately organized as lipospheres (× 2,800).

Male rat exposed to 200 p.p.m. DDT for 6 months. Oil Red O with hematoxylin counterstain after formalin fixation, potassium dichromate mordanting, and paraffin imbedding.

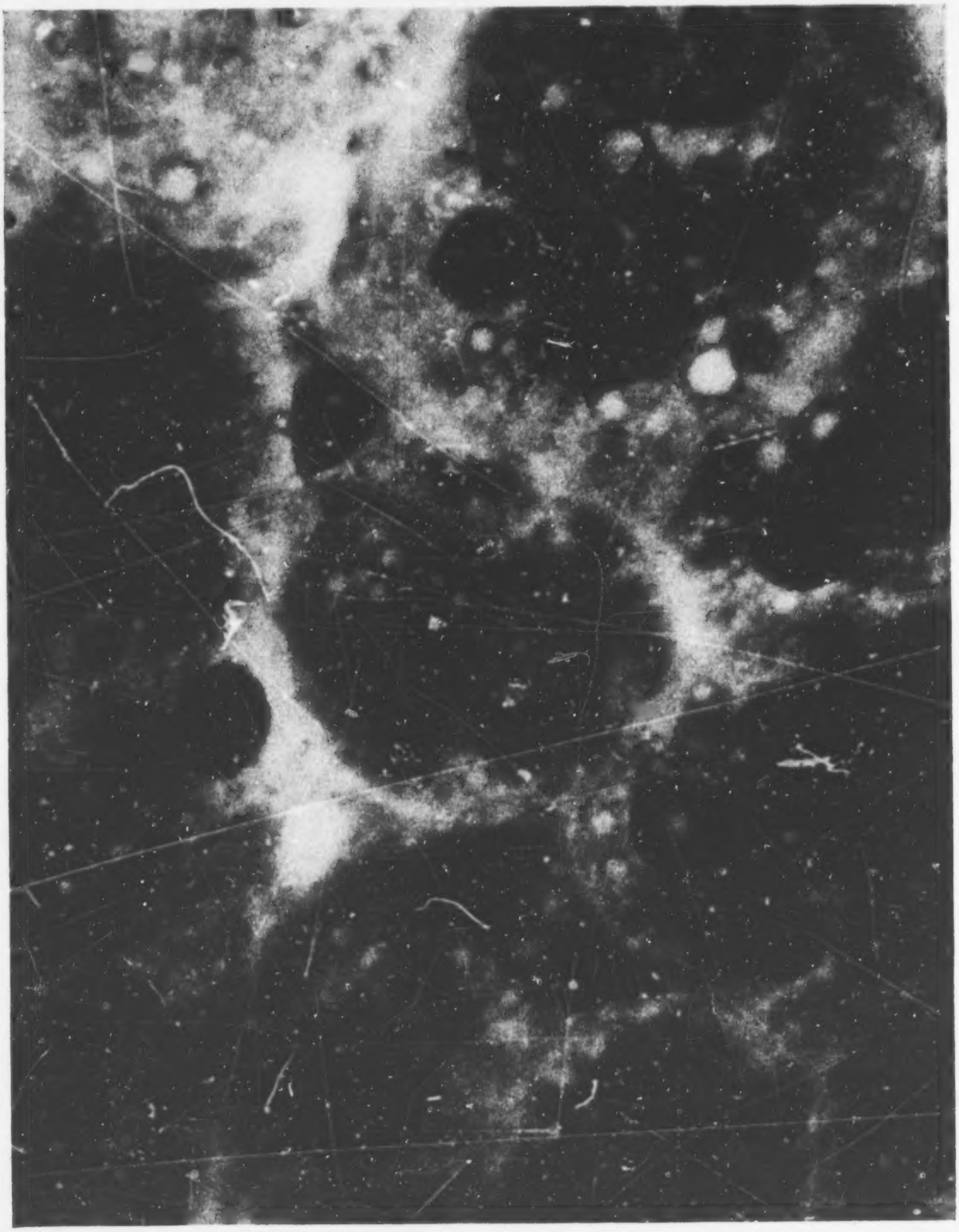


Figure 3. Slight, normal variations in liver cell size and granule content (× 2,800).

Male rat fed on standard Purina diet for 6 months. Azure eosinate after Zenker-formol fixation.

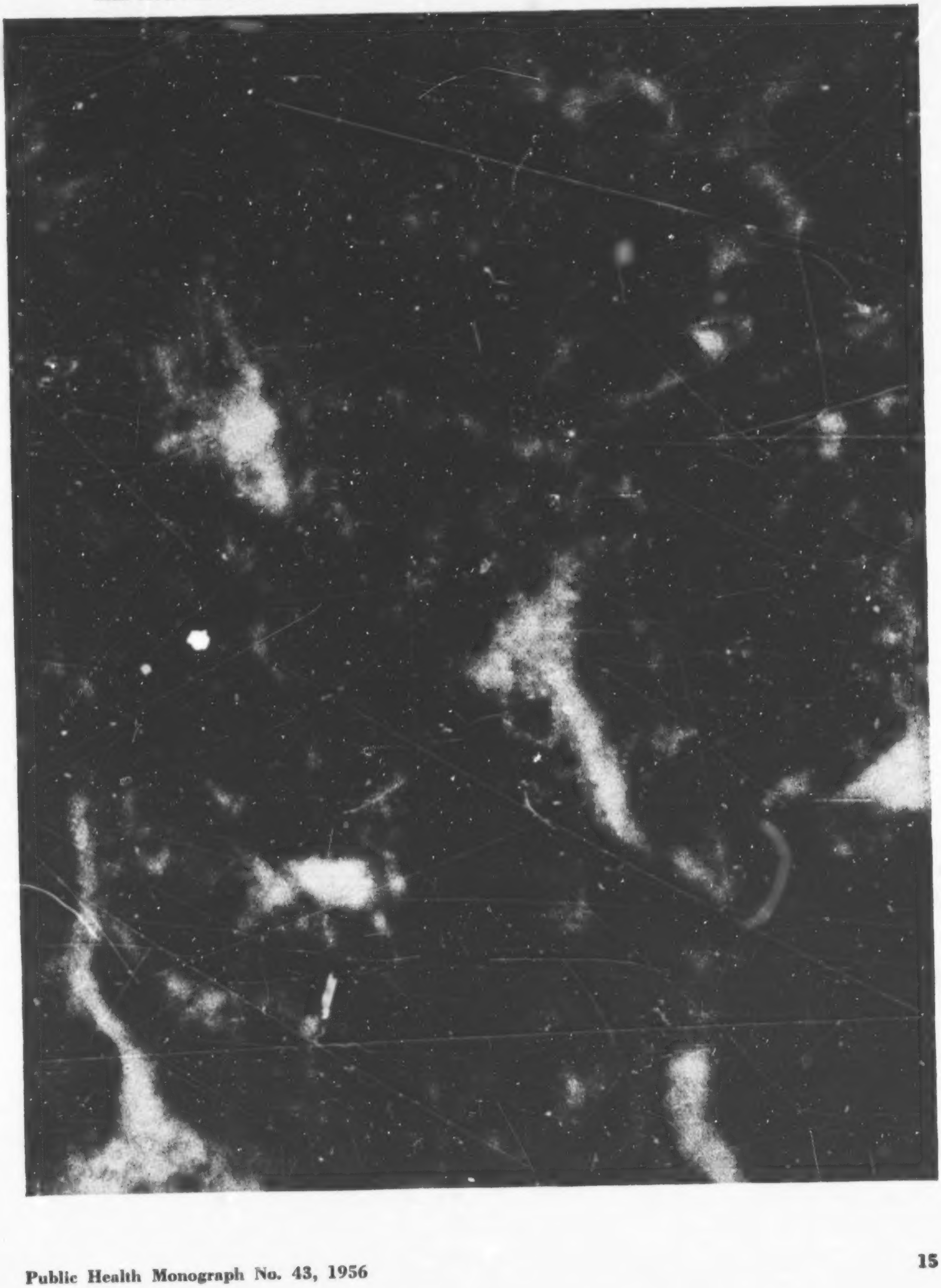


Figure 4. Moderate margination and irregular hypertrophy of liver cells (× 2,800). Note considerable variation from cell to cell at this dosage level.

Male rat fed 50 p.p.m. DDT for 6 months. Azure eosinate after Zenker formol-fixation.

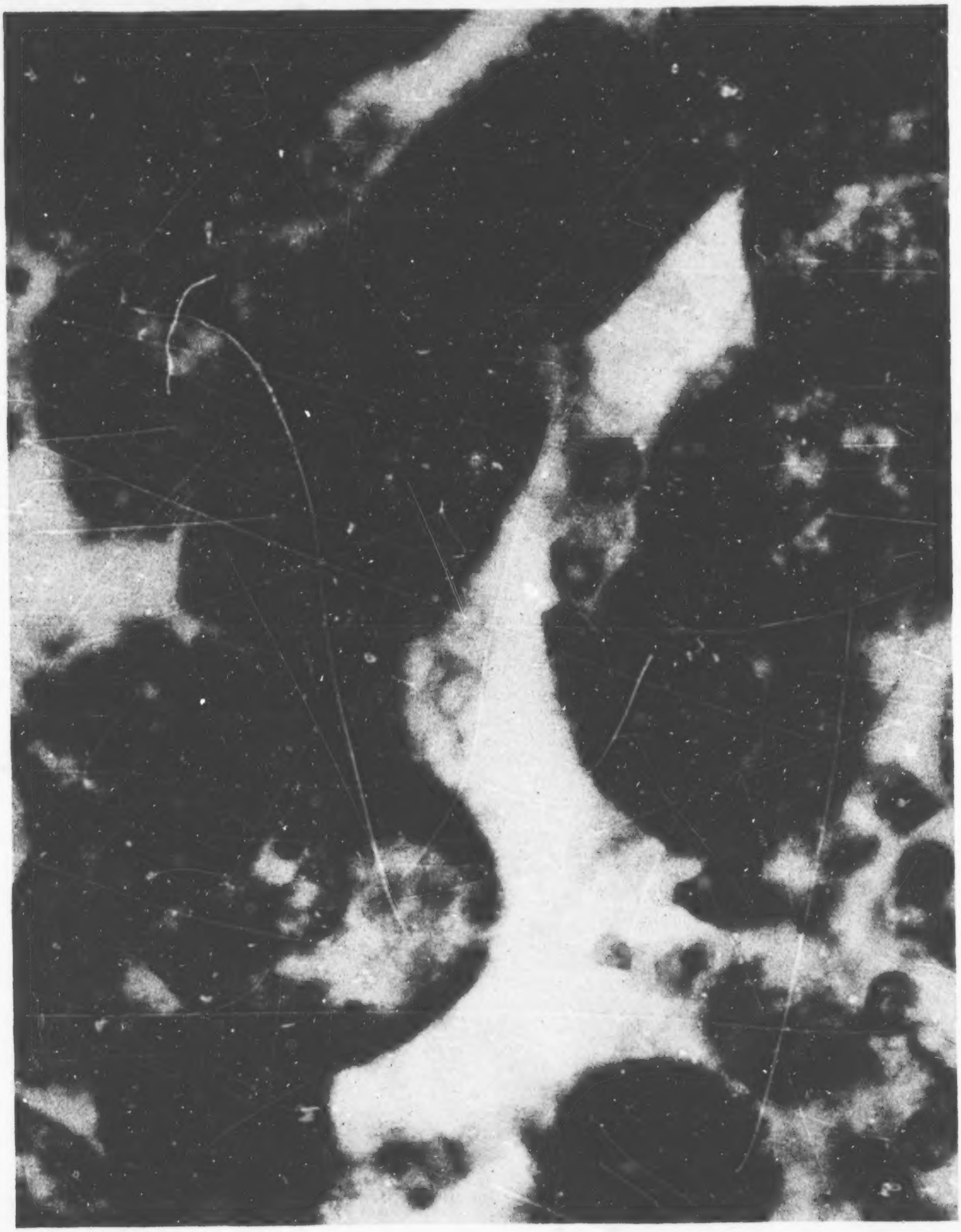


Figure 5. Severe and uniform liver cell margination and hypertrophy (× 2,800). Also demonstrates that large inclusion bodies can be dimly visualized by routine oversight staining techniques.

Male rat fed 200 p.p.m. DDT for 6 months. Azure eosinate after Zenker-formol fixation.

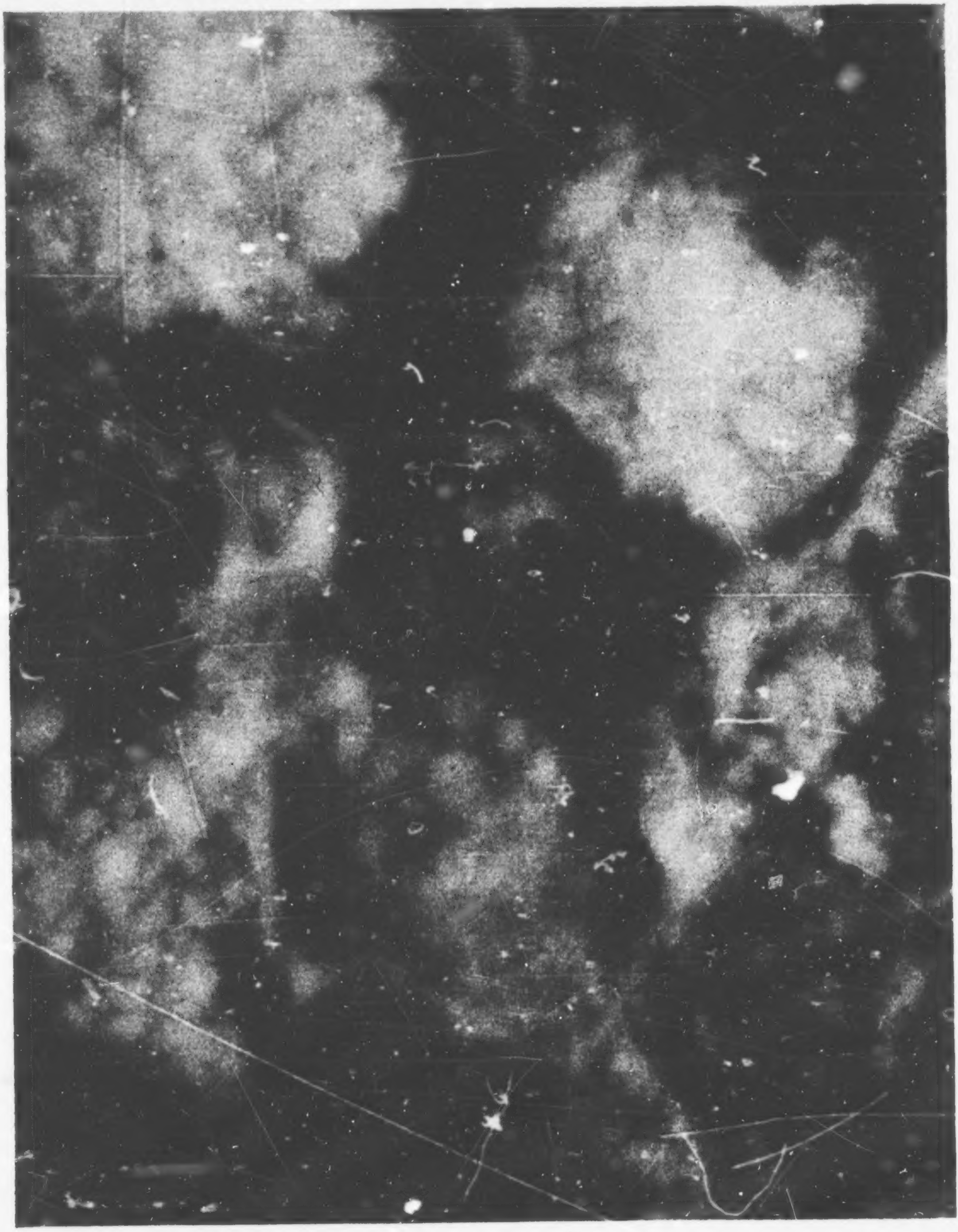


Figure 6. Two lipospheres visible as sharply differentiated bodies against the cytoplasmic background of otherwise normal liver cells. Their simple structure is typical of this degree of poisoning (× 2,800).

Male rat exposed to 5 p.p.m. DDT for 6 months. Aniline acid fuchsin-orange-G-aniline blue after acid Zenker fixation (× 2,800).

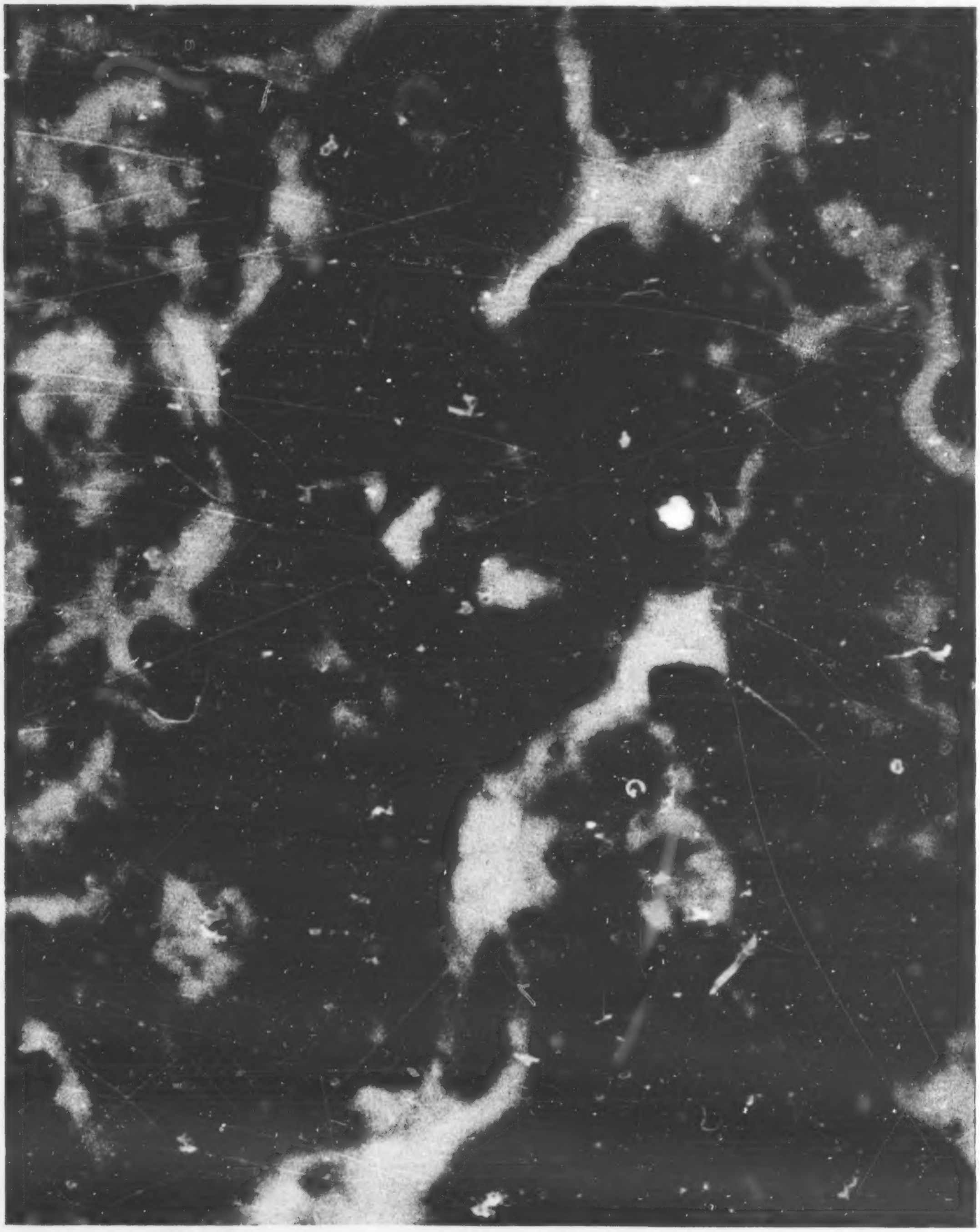


Figure 7. Fully developed lipospheres with complex internal organization and apparent coalescence into large masses. Liver cells also reveal hypertrophy (× 2,800).

Male rat fed 200 p.p.m. DDT for 6 months. Aniline acid fuchsin-orange-G-aniline blue after acid Zenker fixation.

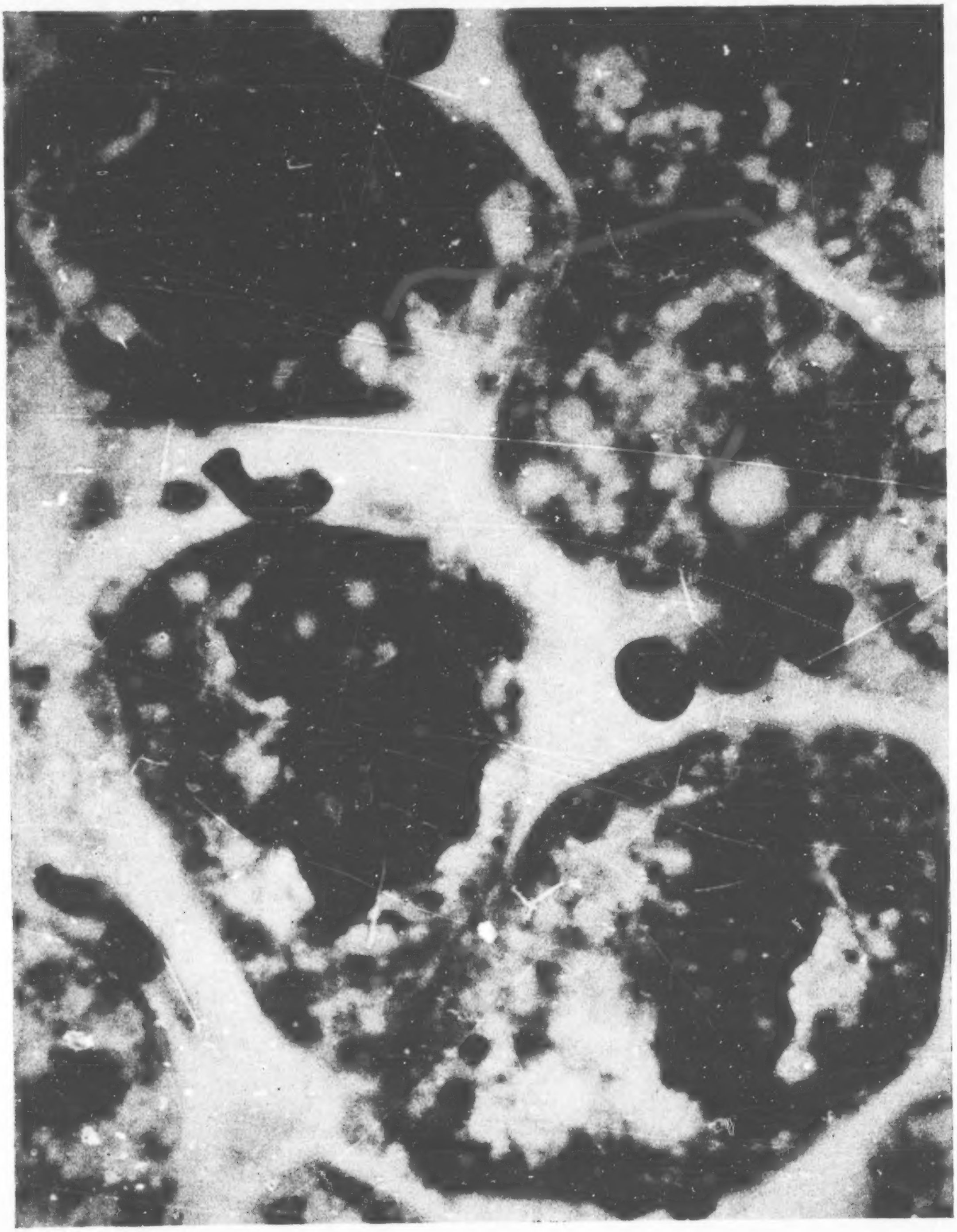
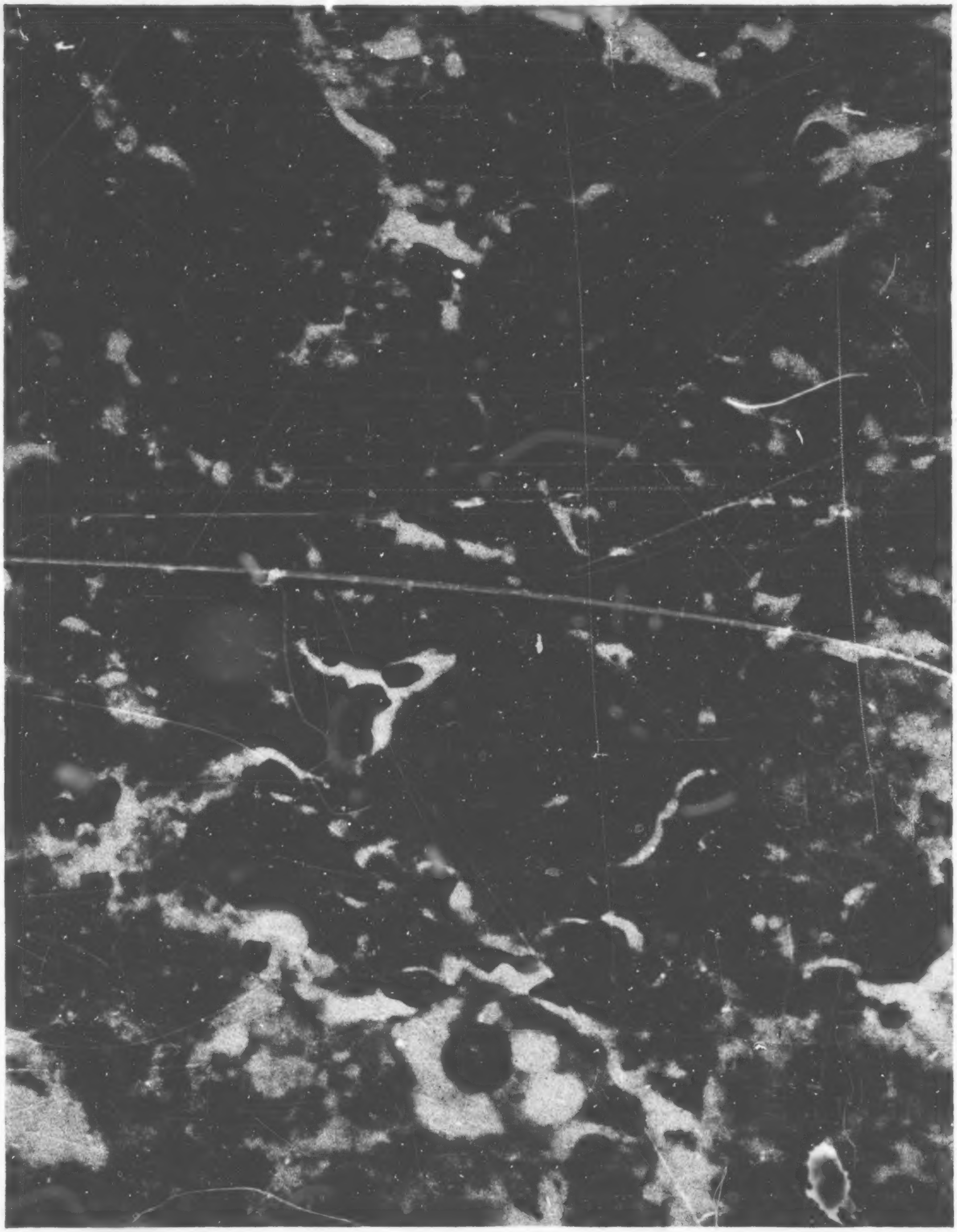


Figure 8. Focus of hyalinization, hydropic vacuolation, and liver cell necrosis with early leucocytic infiltration (× 1,200). This is the most severe lesion demonstrated.

Male rat maintained on 1,000 p.p.m. DDT for 25 days followed by 5 days at 5,000 p.p.m. Hematoxylin and eosin after Zenker-formol fixation.



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The number of lipospheres per cell and the number of affected cells are both increased. The structures tend to coalesce to form pleomorphic masses (up to 25μ in greatest diameter), which may cause considerable displacement of the cell contents. As the liposphere enlarges, many vacuoles, separated by a fine reticulum, replace the single vacuole, whereas the capsule acquires a definitely fibrillar structure with distinct laminations. The capsule stains a brilliant pink with the modified Bensley's aniline acid fuchsin stain. This makes possible the identification of lipospheres even though only a very few are present in an entire liver section. Blood corpuscles, cytoplasmic granules, and nonspecific hyaline granules also take the stain, but with a distinguishable hue. Completely developed lipospheres also may be stained with a variety of other stains, including hematoxylin and eosin, azure eosinate, phosphotungstic acid, iron hematoxylin, fat stains, metallic impregnations, and so on.

The existence of lipospheres has been confirmed and their characteristics studied in living liver cells suspended in Ringer's solution and viewed with the phase microscope. The fixing and staining reactions of the complex structures suggest that the fat in them forms part of a lipoprotein complex. When numerous, lipospheres are uniformly present in the inner half of each lobule, with most of them having a roughly midzonal distribution sparing the innermost ring of cells.

Liver cell necrosis was produced only by feeding rats at 1,000 p.p.m. for 25 days and then at 5,000 p.p.m. for as much as 5 days. Under these conditions, the liver showed severe cytoplasmic hyalinization which progressed to a foamy or hydropic vacuolation, and then, finally, frank necrocytosis (fig. 8). Focal infiltrations of acute inflammatory cells were noted to be replacing the dead liver cells. The peripheral cells showed considerable variation in size, many being shrunken. The fat content appeared to diminish, and a diffuse cytoplasmic reaction replaced the usual distinct globules or granules. Even the fat content of lipospheres appeared depleted. Most of the vacuolar alterations noted were fat free and of the hydropic variety. Lipospheres were characteristic of intact cells and could not be identified within either severely hydropic or necrotic cells.

In grading the pathology found in a particular slide, emphasis was placed on the general over-all picture rather than on local accentuations. Nodule formations similar to those described by Fitzhugh and Nelson (*6*) were observed; the nodules often showed a different morphology from that of the surrounding tissues. However, for most purposes, these focal incongruities were neglected. Each tissue alteration was diagnosed as belonging to 1 of 5 groups varying from normal to extremely advanced.

The results from experiments 1–4 are summarized in table 9. Only changes considered to be in excess of normal variation have been tabulated. Thus, data on the storage of neutral and lipoid fat are not included in table 9 because the extreme variability in these constitutents at any given dosage precluded their useful correlation with dosage levels. It is true that the higher dosages were rather consistently associated with increased fat storage, if for no other reason than the presence of lipospheres. Some variation in the distribution of cytoplasmic granules and in cell size was encountered, of course, in rats exposed to the least possible amount of DDT. Thus, the distinctions between control and exposed rats in regard to margination and hypertrophy were only quantitative. The situation was different with respect to lipospheres. Under the most carefully controlled conditions of exposure (experiment 2, groups 1 and 2), these inclusion bodies were never observed in normal animals. However, where some contamination of the control diet had occurred (experiment 1a, group 1), a few such bodies were found in the liver on very rare occasions.

The following generalizations are indicated: Changes in the liver, clearly distinguishable from the controls, have been observed in all male rats fed 50 p.p.m. or more of DDT for 6 months or more. Liver changes were produced in all female rats fed at levels of 200 p.p.m. or more, but they were generally milder in degree than the changes seen in comparable males. Mild changes could be detected in from one-third to one-half of the males fed 5 p.p.m. and 15 p.p.m. of DDT for 6 months. Thus, the male was far more sensitive to liver cell changes associated with DDT than was the female. In a general way, the intensity of the four kinds of liver changes (fat, margination, hypertrophy, and lipospheres) in either sex was directly proportional to the duration of exposure. The time of earliest appearance differed for different liver changes, but was inversely proportional to dosage in each instance. All of these four changes were reversible.

The generalizations just mentioned were valid in spite of a considerable variation in the liver changes, particularly at low dosage levels. Variation among control animals has already been mentioned. In addition, margination, hypertrophy, and lipospheres occasionally failed to appear individually in rats fed DDT at high levels for 6 months, although no rat under these conditions failed to show at least one of the changes. The combination of hypertrophy and margination is typical of chlorinated hydrocarbon poisoning. Of the more reliable criteria of DDT-induced change, margination appeared most rapidly. Margination was a quantitative test of high sensitivity and only moderate specificity for the male rat. Hypertrophy was a test of high sensitivity and intermediate specificity for the male. When present, lipospheres were most helpful as being characteristic of exposure to DDT. When lipospheres were undetectable, it could not be concluded that exposure had not occurred. This criterion proved highly specific and somewhat sensitive but because of technical difficulties was not a convenient screening method.

The mildness of the observed changes is indicated by the fact that only at 200 p.p.m. and 400 p.p.m. did occasional liver cells show a suggestive depletion of mitochondria, and abnormality of the Golgi apparatus was not

Table 9. Liver cell changes in the rat following ingestion of diets containing different concentrations of DDT (Experiments 1–4)

Kind of observation	Sex	Concentration of DDT in food						
		<0. 06	5	15	50	200	400	1,000
		Margination						
Earliest appearance (month)	Male	>ob	>ob	6	2	2	2	0.25
	Female	>ob	>ob	>ob	>ob	>ob	4–6	NS
Earliest disappearance (month)	Male	------	------	8	8	8	8	NS
	Female						2. 5	NS
Proportion of rats affected	Male	0	0	Few	Most	Most	All	All
Degree of involvement		0	0	Mild	Severe	Severe	Severe	Severe
Proportion of rats affected	Female	0	0	0	0	0	Few	NS
Degree of involvement		0	0	0	0	0	Mild	NS
		Hypertrophy						
Earliest appearance (month)	Male	>ob	>ob	6. 5	2	2	2	. 25
	Female	>ob	>ob	>ob	>ob	4	2	NS
Earliest disappearance (month)	Male	------	------	2	5	8	8	NS
	Female					5	5	NS
Proportion of rats affected	Male	0	0	Few	Most	Most	All	All
Degree of involvement		0	0	Mild	Mild	Severe	Severe	Severe
Proportion of rats affected	Female	0	0	0	0	Most	All	NS
Degree of involvement		0	0	0	0	Mild	Mild	NS
		Lipospheres						
Earliest appearance (month)	Male	>ob	2–6	4–6	2	2	2	0. 5
	Female	>ob	>ob	>ob	>ob	6. 5	6. 5	NS
Earliest disappearance (month)	Male	------	0. 5	0. 5	0. 5	1–5	8–14	NS
	Female					0. 5	2	NS
Proportion of rats affected	Male	0	Half	Third	Most	Most	All	Half
Degree of involvement		0	Mild	Mild	Severe	Severe	Severe	Severe
Proportion of rats affected	Female	0	0	0	0	Rare	Few	NS
Degree of involvement		0	0	0	0	Mild	Mild	NS

>ob—Greater than the period of observation.
NS—Not studied.

observed even at these levels. In animals fed 400 p.p.m. or less, glycogen stores were generally high, a finding consistent with the animals' good state of nutrition.

Experiment 4 indicated that the observed variation in liver cell changes could not be correlated with the quality of DDT (technical or *p,p'*-isomer), or with the degree of storage in the fat or in the liver by individual rats. This was true even though at least one tissue slice was examined from each of the three major liver lobes in each animal.

Discussion

Sarett and Jandorf (*20*) demonstrated that female rats fed DDT at a concentration of 700 p.p.m. for 36 to 40 days showed not only an increase in liver weight but also an increase in the phospholipid of the liver. A similar increase in liver weight and phospholipid content has been found in male and female rats exposed to dieldrin.[8] The capsules of lipospheres take stains which are reactive for phospholipids. The known facts are consistent with the hypothesis that the increased phospholipids which may be demonstrated chemically are the same as those which may be demonstrated histologically in the lipospheres.

As has been frequently demonstrated, cytoplasmic granules are the storage areas of ribosenucleic acid (*22*). Moreover, there is much evidence to correlate the concentration of ribosenucleic acid with protein synthesis, and the great variability in pattern and concentration of these granules under different metabolic conditions is well known (*23–27*). This possibly explains the difficulty in distinguishing minor alterations from the normal physiological patterns.

Hyaline, cytoplasmic inclusion bodies in the liver cells of rats exposed to a variety of compounds have been described. It is clear that a part of these inclusion bodies previously described for animals exposed to DDT are identical with the structures referred to as lipospheres. Dr. Arthur A. Nelson, of the Food and Drug Administration, generously lent tissue blocks. New sections from these blocks, stained by special methods as outlined above, clearly revealed the presence of a very few lipospheres with typical morphology and staining properties. Dr. Joseph F. Treon and Dr. Frank P. Cleveland, of the Kettering Laboratories, lent slides originally reported on by Deichmann and associates (*12*). Even though these slides were stained with a routine oversight stain, many well-developed lipospheres with typical capsules could be seen in them.

It has not been possible to judge objectively whether the hyaline inclusion bodies reported by Lillie and Smith (*1*) and by Lillie, Smith, and Stohlman (*5*) were or were not identical with lipospheres.

Unpublished observations indicate that other chlorinated hydrocarbon insecticides (specifically chlordane, dieldrin, lindane, and toxaphene) are capable of eliciting lipospheres.

Even compounds more remotely related to DDT than the other chlorinated hydrocarbon insecticides may produce lipospheres. Photomicrographs of liver cells of rats exposed to various carcinogenic azo compounds suggest the presence of these cytoplasmic inclusions (*28–30*). Other hyaline spheres have been described by Pappenheimer and Hawthorne (*31*) and by Price and Laird (*32*). Original studies with several azo compounds have confirmed the occasional presence of lipospheres but indicated that most of the hyaline or oxyphilic, cytoplasmic masses induced by these compounds lacked the internal organization diagnostic of these specific inclusions.

The inclusion bodies induced by various chlorinated naphthalenes and so distinctly figured by Bennett and associates (*33*) certainly resemble lipospheres, but no original investigation has been made of the matter.

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[8] Durham, W. F., and Hayes, W. J., Jr.: The toxicology of dieldrin. III. The effect of dermal application on liver biochemistry in the rat. (In preparation.)

In regard to the specificity of lesions associated with chlorinated hydrocarbon insecticides, the presence together of hypertrophy, margination, and lipospheres is highly characteristic. However, each of these types of pathology can be found as the result of quite different stimuli. For instance, poisoning with lead acetate or mercuric acetate can produce a marked degree of margination without necessarily producing the other lesions. Lead can produce inclusion bodies, but these are quite different from lipospheres in structure and location.

Azo compounds can produce all of these lesions, but their distribution tends to be somewhat erratic and highly variable from lobe to lobe. Moreover, azo compounds produce bile duct proliferation, necrocytosis, pigment deposition, cirrhosis, and atypically hypertrophied liver cells. A marked clumping and hyalinization of the cytoplasm also helps to distinguish the effect of azo dyes from that of the chlorinated hydrocarbon insecticides. Elemental phosphorus can produce margination and hypertrophy, but again these changes lack the uniform lobular distribution found with DDT, and severe necrocytosis is much more common with phosphorus. Hence, the lesions produced by chlorinated hydrocarbon insecticides are marked more by their association and by a lack of other or more severe forms of liver disease than by any specificity of each lesion alone.

The results reported in this paper suggest that even routine methods of tissue preparation and staining should reveal liver changes in rats fed DDT at the higher levels used by Cameron and Cheng (*9*). No explanation for their failure is clearly suggested although, as previously mentioned, the possibility has not been ruled out that their method of administering DDT may have accounted for the reported absence of liver changes. In any event, the absence of significant changes in the tissues studied by Cameron and Cheng is provisionally accepted as a fact.

As noted above, some other workers have failed to observe changes. Of the four changes, lipospheres are most likely to be absent. Very few lipospheres were found in the tissue supplied by Dr. Nelson. Even during the preparation of this paper, Dr. John M. Barnes [7] has indicated in a personal communication that an attempt to produce lipospheres with DDT has been unsuccessful in his laboratory. It thus appears that, although the liver cell changes under discussion depend on DDT dosage, they also may depend on some secondary factor, the existence and the character of which remain to be learned.

There can be no doubt regarding the reality of the morphological changes in the rat liver reported in this paper and in some other papers already cited. The physiological interpretation of the changes when they do occur is not so clear. The changes (exclusive of necrosis) have been regarded by certain authors as an injury produced by DDT, but the possibility that they represent an adaptation is not ruled out by available information. The fact that the changes are generally proportional to dosage is consistent with either view. There is no direct evidence that the changes are adaptive except, perhaps, that rats which develop signs of intoxication when fed 200–400 p.p.m. generally show spontaneous improvement in spite of continued dosage. This improvement is accompanied by development of the liver changes and not their regression. In any event, the following facts should be considered in evaluating the observed liver alterations:

1. The hepatic changes are restricted to rodents (*8*) although the symptomatology of DDT poisoning is similar in a wide range of species.
2. Even in rodents, the mild liver changes (fat deposition, margination, hypertrophy, and liposphere formation) do not parallel symptomatology. These changes appear unrelated to the liver cell necrosis produced by large doses of DDT and, be that as it may, in severe poisoning in all species it is not the function of the liver but the function of the neuromuscular system which is severely and decisively affected.
3. The characteristic changes are reversible and appear to be a direct response to stimulus; their temporary persistence when DDT is withdrawn from the diet is adequately explained by the continuous exposure of the tissues to DDT being mobilized from endogenous stores.

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[7] Toxicology research unit, Serum Research Institute Woodmansterne Road, Carshalton, Surrey, England.

4. The mild liver changes are much more prominent in male than in female rats; this is in contrast to the slightly greater susceptibility of the female rat to DDT poisoning and to the markedly greater storage of DDT by the female.

5. The presence of characteristic liver changes is consistent with normal liver function as revealed by the BSP test. The development of lipospheres, the increase in liver weight, and the increase in phospholipid content of the liver may be different aspects of the same change.

6. The presence of characteristic changes in a liver cell is consistent with the presence of abundant mitochondria and a well developed Golgi apparatus, both structures being admittedly highly labile and sensitive.

Summary

1. A total of 178 male and 104 female white rats were fed dietary levels of DDT ranging from less than 0.05 to 5,000 p.p.m. for periods ranging from 1 week to 14 months. Recovery was also followed in some of them.

2. Mortality was not appreciable, and no effect on growth was observed on rats fed levels of 400 p.p.m. or less.

3. Liver weight was definitely increased in rats fed 400 p.p.m.; but there was no evidence of increase at 200 p.p.m. or less. Bromsulphthalein excretion by the liver was not affected in rats fed 400 p.p.m. or less.

4. Rats stored DDT in proportion to dosage. Both sexes convert a portion of ingested DDT to DDE, but female rats store more DDT and much more DDE than males. DDT-derived material is gradually lost when dosage is discontinued.

5. Levels of 400 p.p.m. or less produced histological changes in the liver only. The changes in the parenchymal cells of the liver consisted of an increased deposition of fat, margination of cytoplasmic granules, hypertrophy of the cells, and—most characteristic—the formation of complex, lipoid, cytoplasmic inclusion bodies termed "lipospheres." Changes could be recognized in some male rats at levels of 5 p.p.m. and 15 p.p.m., but in females only at 200 p.p.m. Increased severity of changes was noted with increasing dosage. Necrosis occurred with dosage in excess of 1,000 p.p.m. but not at lower levels.

6. The authors are in general agreement with Laug and associates in regard to the presence of cellular alterations in the liver of rats fed DDT at levels as low as 5 p.p.m. in the diet. However, it is possible that occurrence of the changes depends not only on DDT but on some unidentified secondary factor. Furthermore, it is suggested that greater stress be placed on the mildness of these changes when they do occur, on their ready reversibility, and on their imperfect correlation with liver function or with the clinical effects of DDT. The changes under discussion seem to occur in rodents only; their significance, however, even for rats, is far from clear.

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